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Abstract: E-26 transformation-specific (ETS) proteins are transcription factors directing gene expression through their conserved DNA binding domain. They are implicated as truncated forms or interchromosomal rearrangements in a variety of tumors including Ewing sarcoma, a pediatric tumor of the bone. Tumor cells express the chimeric oncoprotein EWS-FLI1 from a specific t(22;11)(q24;12) translocation. EWS-FLI1 harbors a strong transactivation domain from EWSR1 and the DNA-binding ETS domain of FLI1 in the C-terminal part of the protein. Although Ewing cells are crucially dependent on continuous expression of EWS-FLI1, its regulation of turnover has not been characterized in detail. Here, we identify the EWS-FLI1 protein as a substrate of the ubiquitin-proteasome system with a characteristic polyubiquitination pattern. Using a global protein stability approach, we determined the half-life of EWS-FLI1 to lie between 2 and 4 h, whereas full-length EWSR1 and FLI1 were more stable. By mass spectrometry, we identified two ubiquitin acceptor lysine residues of which only mutation of Lys-380 in the ETS domain of the FLI1 part abolished EWS-FLI1 ubiquitination and stabilized the protein posttranslationally. Expression of this highly stable mutant protein in Ewing cells while simultaneously depleting the endogenous wild type protein differentially modulates two subgroups of target genes to be either EWS-FLI1 protein-dependent or turnover-dependent. The majority of target genes are in an unaltered state and cannot be further activated. Our study provides novel insights into EWS-FLI1 turnover, a critical pathway in Ewing sarcoma pathogenesis, and lays new ground to develop novel therapeutic strategies in Ewing sarcoma.

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Proteasomal degradation of the EWS-FLI1 fusion protein is regulated by a single lysine residue

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Abstract

E-26 transformation specific (ETS) proteins are transcription factors directing gene expression through their conserved DNA-binding domain. They are implicated as truncated forms or interchromosomal rearrangements in a variety of tumors including Ewing sarcoma, a pediatric tumor of the bone. Tumor cells express the chimeric oncoprotein EWS-FLI1 from a specific t(22;11)(q24;12) translocation. EWS-FLI1 harbors a strong transactivation domain from EWSR1 and the DNA-binding ETS domain of FLI1 in the C-terminal part of the protein. Even though Ewing cells are crucially dependent on continuous expression of EWS-FLI1, its regulation of turnover has not been characterized in detail.

Here, we identify the EWS-FLI1 protein as a substrate of the ubiquitin proteasome system with a characteristic poly-ubiquitination pattern. Using a Global Protein Stability approach, we determined the half life of EWS-FLI1 to lie between 2h-4h whereas full length EWSR1 and FLI1 were more stable. By mass spectrometry, we identified two ubiquitin acceptor lysine residues of which only mutation of K380 in the ETS domain of the FLI1 part abolished EWS-FLI1 ubiquitination and stabilized the protein posttranslationally. Expression of this highly stable mutant protein in Ewing cells, while simultaneously depleting the endogenous wild type protein, differentially modulates two subgroups of target genes to be either EWS-FLI1 protein dependent or turnover dependent. The majority of target genes is in an unaltered state and cannot be further activated.

Our study provides novel insights into EWS-FLI1 turnover, a critical pathway in Ewing sarcoma pathogenesis and lies new grounds to develop novel therapeutic strategies in Ewing sarcoma.

Introduction

E-26 transforming specific (ETS) family members are strong activators or repressors of transcription with a highly conserved ETS domain (1-3). ETS transcription factors (TFs) bind most commonly in complexes to a GGA core region in order to mediate gene expression (4,5). Their main biological functions include regulation of differentiation, lineage determination of the hematopoietic system and control of angiogenesis (6,7). Most of the ETS family members have oncogenic potential, since truncated or overexpressed ETS proteins have been linked to several cancer entities (8-11). ERG or ETV1 are

frequently fused to the TMPRSS2 promoter in prostate cancer, whereas ETV1 and ETV6 are implicated in leukemia (12,13). Like other aberrant fusion proteins, they act as drivers of uncontrolled cell growth and survival (14,15). However, most TFs do not harbor an enzymatic pocket and are therefore difficult to target directly. Novel strategies which uncover vulnerable sites in TFs are urgently needed to develop novel targeted therapies (16).

Ewing sarcoma is a rare pediatric bone and soft tissue tumor with an aggressive behavior and prevalence to metastasize (17,18). Its main genetic abnormalities are EWS–ETS rearrangements, among them most commonly the EWS gene on chromosome 22 fused to FLI1 on chromosome 11 which results in expression of the chimeric transcription factor EWS-FLI1 (19-21). Continuous expression of the fusion protein is crucial for tumor formation, progression and maintenance (22,23) and its downregulation inhibits proliferation and reduces tumor cell growth (24-26). EWS-FLI1 is thought to function mainly as modulator to activate and repress a wide range of target genes, but also to regulate splicing processes or being a component of large interaction networks (27-31). However, inhibition of a single downstream target gene has not been proven effective yet for Ewing sarcoma therapy.

The turnover of most intracellular proteins is mediated via the ubiquitin proteasome system (UPS) which triggers protein degradation (32). Several ETS proteins can be poly-ubiquitinated by different E3 ligases and subsequently degraded by the proteasome (33-36). Considering their high conservation, proteasomal degradation is likely to be the main mechanism of turnover for most ETS family members. Most interestingly, truncated ERG and ETV1 are lacking the N-terminal E3 binding domain which results in a delayed turnover of aberrant ETS proteins (34, 36). Here, we focus specifically on the turnover of the fusion protein EWS-FLI1 which only harbors the ETS and the C-terminal domain from FLI1. The impact on the turnover of this domain fused to N-terminal region of EWSR1, has not been investigated yet. Hence, EWS-FLI1 proteasomal turnover represents a so far uncharacterized mechanism in Ewing sarcoma tumorigenesis, even though EWS-FLI1 degradation has already been linked to the lysosomal pathway (37). Moreover, the exact lysine acceptor residues for poly-ubiquitination have not yet been identified for any wild type or truncated ETS protein.

In the present study, we demonstrate that EWS-FLI1 is predominantly a proteasomal substrate with an unexpectedly high turnover rate mediated by poly-ubiquitination at a single lysine residue. Surprisingly, expression of this highly stable mutant protein in Ewing cells specifically induced subgroups of high-level expressed or turnover sensitive target genes, while the majority of target genes are unaltered.

Hence, our study provides novel insights into EWS-FLI1 turnover suggesting that deflecting its stability could contribute to new therapeutic concepts in Ewing sarcoma.

Results

EWS-FLI1 turnover is proteasome dependent. Since EWS-FLI1 expression is crucial for tumor cell survival (24-26), we were interested to analyze degradation and turnover rate of the fusion protein. To address this, we first used a panel of six different inhibitors targeting proteasomal, lysosomal and autophagosomal protein degradation pathways. Incubation with 20μM of the various inhibitors resulted in a 2-fold upregulation of EWS-FLI1 protein levels only upon MG-132 and Bortezomib treatment, both inhibitors of the chymotrypsin-like proteasome activity (Fig. 1A). We next confirmed this result by selectively inhibiting the proteasomal and lysosomal degradation pathways with two compounds in three different Ewing sarcoma cell lines. Incubation with 20μM of the proteasome inhibitor MG-132 resulted in stabilization of endogenous EWS-FLI1 fusion protein by at least 2-fold while this was not the case for the lysosomal inhibitor chloroquine (Fig. 1B). P27 and LAMP1 were used as positive controls and showed upregulation after treatment as expected. In addition, stabilization of the fusion protein in Ewing cells by MG-132 increased over a 2h-8h treatment period in a time- dependent manner (Fig. 1C). Hence, degradation of EWS-FLI1 is primarily proteasome dependent under steady-state conditions. However, incubation with 20μM cycloheximide (CHX) had only limited reducing effect on the endogenous fusion protein in the Ewing cells over the same 8h time period (Fig. 1D).

Next, we investigated whether the fusion protein is ubiquitinated. To this end, 3xflag-EWS-FLI1 was co-expressed with HA-ubiquitin in HEK293T cells and immunoprecipitated after 48h using anti-flag antibody. Western blotting with an anti-HA antibody revealed at least four distinct ubiquitin bands for EWS-FLI1 which could not be increased by prior treatment with MG132 (Fig. 1E), suggesting a constant ubiquitination of the fusion protein. We next co-expressed 3xflag-EWS-FLI1 with wild type HA-ubiquitin or ubiquitin mutants which are deficient for K48- and K63-linked chains. Immunoprecipitation of the ubiquitinated 3xflag-EWS-FLI1 revealed mainly a decrease of the ubiquitination pattern using K48R HA-ubiquitin (Fig. 1F) indicating that this is the major, but not only, linked ubiquitin chains of the fusion protein

To further validate the fusion protein turnover and to overcome the limitations of classical CHX treatment to determine protein half lives, we next used the Global Protein Stability (GPS) approach (38) (Fig. 2A). Briefly, HEK293T cells were transduced with a reporter construct DsRed-IRES-EGFP in which EGFP is fused to EWS-FLI1. The ratio EGFP/DsRed was determined by FACS and represents a measure for protein stability. Known degron motifs with distinct half lives were used as internal standards and allowed to estimate the half life of EWS-FLI1 to be between 1h and 4h (Fig. 2B). Incubation with MG-132 shifted the ratio closer to 4h, confirming that EWS-FLI1 is a substrate of the proteasome system with a high turnover rate. Additionally, we treated cells with the nuclear export inhibitor leptomycin B (LMB) for 4h and 8h. Treatment with 50nM LMB stabilized EWS-FLI1 in a time-dependent manner (Fig. 2C) indicating that the proteasomal degradation mainly occurs in the cytosol. Similar results were obtained in transiently or stably transduced Ewing cells (Fig. 2D-E), suggesting that the high turnover of EWS-FLI1 is mostly independent of the cellular background. Interestingly, incubation with 20µM CHX again displayed a limited effect on the EGFP/DsRed ratio of EWS-FLI1 reporter construct (Fig. 2B,2E) indicating that classical CHX barely reflects the steady- state turnover rate of the EWS-FLI1 protein and possibly other proteins.

Taken together, our results indicate that EWS-FLI1 is a poly-ubiquitinated protein with an unexpectedly high turnover rate in Ewing sarcoma cells.

EWS-FLI1 turnover depends on one critical lysine residue. To better understand fusion protein turnover, we aimed to identify the lysine residue(s) that are important for degradation. To this, we purified large amounts of 3xflag-EWS-FLI1 or a dominant-negative R386N mutant (39,40) from HEK293T cells. The inactive mutant was included as the inability to bind DNA increases the possibility for ubiquitination. Both samples were then enzymatically digested and subjected to mass spectrometry to identify peptide shifts due to covalently attached ubiquitin. This identified two peptides with the characteristic glycine-glycine shift including residues K298 and K380 (Fig. 3A, supplementary S1A). To determine whether both sites are important for fusion protein turnover, we mutated them individually to arginine. After co-expressing the mutant EWS-FLI1 with HA-ubiquitin and immunoprecipitation from A673 Ewing cells, we found that the K298R mutant was still ubiquitinated comparable to the wild type, while the K380R lost this characteristic pattern (Fig. 3B). In addition, transient expression of the mutants in both A673 and SKNMC Ewing cells revealed that the K380R and the double mutant K298/380R were 2.5-fold, 3.5-fold respectively, more stable than wild type or the K298R mutant (Fig. 3C-D). As wild type EWS-FLI1, also the K380 mutant was still localized in the nucleus (Fig. 3E). We then investigated the role of both lysines in proteasomal turnover by GPS. The half life of the K298R was comparable to wild type EWS-FLI, while K380R shifted close to 24h (Fig. 3F). Wild type EWS-FLI1 and the K298R could be stabilized by an additional incubation with MG-132, however the EGFP/DsRed ratio of the K380R mutant did not change upon treatment (Fig. 3G). Hence, our findings suggest that K380 is the major site required to regulate EWS-FLI1 protein turnover by the proteasome.

EWS-FLI1 fusion protein and wild type FLI1 share a unique site for turnover. To investigate whether turnover by K380 is unique to the fusion protein, we subsequently compared stability and ubiquitination pattern to the full length proteins EWSR1 and FLI1. Surprisingly, both wild type proteins were more stable with half

lives of >4h (FLI1) and close to 24h (EWSR1) (Fig. 4A). While FLI1 could be stabilized by incubation with MG-132 (or destabilized by CHX), EWSR1 did not change its already long half life by these treatments (Fig. 4B). As FLI1 was clearly poly-ubiquitinated similar to the fusion protein, EWSR1 showed strong mono- and di-ubiquitination (Fig 4C). Next, we mutated the previously identified lysine residue to arginine (FLI1: K334). By GPS, the K334R also increased its half life towards 24h (Fig. 4D). Additionally, mass spectrometry of purified 3xflag-FLI1 revealed glycine-glycine shifts for peptides containing lysines K172 and K252 (peptide spectra in supplementary S1B-C). Whereas K172 is located in the N-terminal part and therefore not present in the fusion protein, K252 corresponds to the EWS-FLI1 K298 residue. Single arginine mutants of both residues display EGFP/DsRed ratios comparable to wild type FLI1 (Fig. 4D, dotted lines) indicating that both fusion and full length protein turnover is regulated by one single lysine residue. Indeed, incubation of FLI1 and K334R with MG-132 only shifted the wild type, but not the K334R mutant ratio (data not shown). Interestingly, the K334/K380 residue is conserved within most ETS family members (Fig. 4E).

Together, our findings indicate that, despite differences in turnover, stability of both EWS-FLI1 and wild type FLI1 is regulated by the same critical conserved residue in the ETS domain.

EWS-FLI1 target genes expression is subdivided into three major groups. Modulation of EWS-FLI1 target gene signature is one of the best studied mechanisms in Ewing sarcoma. EWS-FLI1 is activating a wide range of target genes like NKX2.2, NR0B1 or IGF1 (30,31,41-43), but has also repressive functions as shown for IGFBP3, PHLDA1 and LOX (44-46). Consequently, this is thought to be critical for oncogenic properties as depletion of the fusion protein results in growth inhibition and cellular senescence (24,47). To better understand the effect of EWS-FLI1 stability on the target gene signature, we established inducible exchange cell lines which were capable to knock down the endogenous EWS-FLI1 while simultaneously expressing 3xflag-EWS-FLI1 or the K380R mutant. To this, we first sorted cells expressing these flag tagged overexpression constructs by flow cytometry for the same low EGFP expression (the selection marker of the pInducer21 vector) to obtain a homogeneously expressing population (Fig. 5A). We then subsequently transduced each of these clonal pools with two different shRNA constructs against the 3'UTR of EWS-FLI1 (Fig. 5B). Upon doxycyline induction for 48h, endogenous EWS-FLI1 was exchanged with ectopic wild type or mutant 3xflag-EWS-FLI1versions (Fig. 5C). As observed before, the K380R mutant stabilized the protein posttranslationally by at least four-fold (Fig. 5C) while EWS-FLI1 mRNA levels were comparable as shown for shEF#2 (Fig. 5D, not shown for shEF#1 based cell lines). We then extracted total RNA for microarray expression profiling and analyzed the EWS-FLI1 target genes signature for their differential expression upon stabilized fusion protein (supplementary S2). A total of 497 probe sets were downregulated by at least 1.5-fold due to EWS-FLI1 depletion from which 250 could be rescued to at least normal levels by induction of EWS-FLI1 wild type (Fig. 6A-B). Only if the EWS-FLI1 target genes were modulated and recovered in the paired comparison of EWS-FLI1 induction and knockdown at each 24h and 48h, they were considered as rescued. This pattern was similar for repressed target genes as 333 probe sets were at least 1.5-fold upregulated from which 164 could be again repressed by ectopic EWS-FLI1 (Fig. 6A, 6C). However, we were most interested in the target genes which were further modulated with stabilized protein levels. Surprisingly, the large majority of these genes appeared to be unmodified and displayed no difference in target gene expression between wild type and mutant EWS-FLI1. However, two subgroups were modulated differentially by the mutated fusion protein. One subgroup seems to be protein sensitive as 15 individual genes displayed higher RNA levels by at least 1.5-fold upon expression of stabilized EWS-FLI1 protein. From the other subgroup, 29 rescued candidates were fusion protein turnover sensitive and could only partly be modulated by the mutant in comparison to wild type EWS-FLI1 (Fig. 6A-B, list supplementary S2). Among the protein sensitive candidates, IGF1 and long non-coding RNA EWSAT1 have been already characterized (41,48) while most other target genes have not been implicated in EWS-FLI1 oncogenesis. Selected target genes from the protein dependent and unmodified group were validated by quantitative RT-PCR level for two

different shEWS-FLI1 sequences (Fig. 6D, shown for shEF#2). Even though the exact mechanism of EWS-FLI1 gene repression is not fully understood, the pattern of target gene modulation resembled that of activated target genes. The majority of repressed genes were unmodified, but also here two subgroups differentially modulated repression upon expression of stabilized protein levels (Fig. 6C, 6E, list supplementary S2).

Taken together, our results indicate that 82% of activated, and 93% of repressed target genes have unaltered levels and cannot be further increased by higher EWS-FLI1 protein levels. Only subsets of the target gene signature are differentially modulated implying that stability of EWS-FLI1 directs these important subgroup or primarily modulates other mechanisms.

Discussion

Here, we demonstrated that EWS-FLI1 is a polyubiquitinated substrate that is primarily degraded by the proteasome. The high turnover of the fusion protein is controlled by one critical lysine residue located in the conserved ETS domain. By exchanging the wild type EWS-FLI1 with its turnover deficient mutant, we could show that protein stabilization differentially modulates two subsets of target genes whose expression could either be enhanced (protein dependent) or only partially rescued (turnover dependent). However, the majority of target genes are in an unmodified state and independent of protein stabilization.

Attachment of ubiquitins to a substrate triggers a variety of outcomes including degradation and signaling depending on the ubiquitin acceptor site and ubiquitin chain linkage (32). Here, we identified the fusion protein EWS-FLI1 as a substrate of the proteasome system in a time and dose dependent manner. The previously suggested lysosomal degradation route (37) possibly contributes to turnover, but appears less relevant compared to the proteasomal pathway under steady state conditions.

During the last decades, a variety of different technologies have been developed to dissect and understand ubiquitin mediated processes (49). GPS profiling was initially established to identify novel proteasomal or cullin-RING ligase substrates (38,50,51). Thus, we applied this approach to analyze EWS-FLI1 turnover. Displaying the EGFP/DsRed ratio for the fusion protein and known standard reporter constructs, we confirmed EWS-FLI1 turnover as proteasome dependent and mapped the half life to be between 1h-4h. As most potent transcription factors, also EWS-FLI1 displays a fast turnover (52,53). Using Leptomycin B, we could show that EWS-FLI1 is indeed exported out of the nucleus for degradation suggesting that the fusion protein tightly keeps balance of its cytosolic proteolysis. The apparent exclusive nuclear localization might therefore be due to its rapid cytosolic degradation.

However, incubation of cells with the translation synthesis inhibitor CHX barely reflected the high turnover observed in the GPS under steady-state conditions. Using this assay, others already suggested EWS-FLI1 as a stable protein (37). In contrast, by using antisense oligodeoxynucleotides (26) and RNAi approaches, it is known that the fusion protein can be efficiently downregulated within a short 24h time frame which would not be possible with a highly stable protein. The discrepancy between the GPS/RNAi data and CHX treatment therefore remains. However, complete inhibition of protein synthesis by CHX also affects other components of the ubiquitin system such as E2 conjugating enzymes or E3 ligases. Possibly, if a required E3 ligase is unstable and immediately depleted, EWS-FLI1 turnover might be prolonged as seen here with classical CHX treatment.

While EWS-FLI1 has been shown to be posttranslationally modified (54-56), ubiquitination remained unknown. Here, we demonstrated that the fusion protein is polyubiquitinated with a clear and distinct pattern which supports the concept of a constant turnover. To better understand how the ubiquitinated fusion protein is degraded, we used affinity purified EWS-FLI1 for mass spectrometry and searched for glycine-glycine modifications in the fragmented peptide sequence. From two possible ubiquitin acceptor sites, only mutation of the K380 clearly abolished ubiquitination and posttranslationally stabilized the protein. While focusing on EWS-FLI1 degradation, we do not exclude that this critical lysine may also trigger non-proteolytic signaling via alternative linked polyubiquitin chains. It is also worth to mention here that we have analyzed our mass spectrometry data for occurrence of glycine-glycine shifts of the ubiquitin moieties. We have identified peptide

shifts mainly for K48-, but also K63-linked ubiquitins (data not shown) confirming our findings that K48-linked chains are highly abundant, but not the exclusive EWS-FLI1 ubiquitination pattern. Other ubiquitin linked chains or mixed chains might also be relevant.

Further, mutation of this residue may also inhibit other modifications such as sumoylation. Acetylation of this residue has not been detected in contrast to the K298 site (55).

The comparison of EWS-FLI1 with its full length proteins revealed that the polyubiquitin pattern is similar to FLI1. Indeed, mutation of the corresponding lysine residue in the ETS domain posttranslationally stabilizes both proteins. As the K380/K334 residue is highly conserved among most ETS family members, it might contribute to proteasomal degradation in other ETS proteins and their fusion proteins. Most interestingly, EWS-FLI1 displays a higher turnover compared to its full length proteins EWSR1 and FLI1. In contrast, the ETS proteins ERG or ETV1 which are fused to the TMPRSS2 promoter in prostate cancer stabilize the protein and confer a physiological advantage to cancer cells (33,34,36). The truncated protein versions display increased stability due to loss of the N-terminal E3 ligase motif. However, EWS-FLI1 is not a truncated version of FLI1, instead the N-terminal domain is dominated by EWSR1 with a stronger transactivation domain. If the turnover and subsequent E3 ligase binding of ETS members is conserved, EWS-FLI1 might possibly interact with E3 ligases via its EWSR1 domain giving rise to a new regulatory mechanism. However, the basic concept that truncated oncogenic TFs are more stable than their wild type counterparts (36) might not apply for fusion proteins consisting of two unrelated protein domains. Whether this is indeed a general paradigm or rather specific for EWSR1 related fusion proteins needs to be further elucidated.

We then deciphered the role of EWS-FLI1 turnover in transcriptional regulation of target genes. To investigate the influence of stabilized EWS-FLI1 on target gene expression, we established an inducible Ewing cell system allowing to deplete endogenous EWS-FLI1 protein while simultaneously expressing the wild type fusion protein or a turnover deficient mutant. This system largely circumvents interference with endogenous fusion protein, enabling us to study the dynamic behavior of posttranslationally stabilized EWS-FLI1 protein on transcriptional regulation. Surprisingly, around 85% of both activated and repressed target genes are in an unaltered state including well characterized target genes such as NR0B1 or STEAP1 (30,57).

Even though our EWS-FLI1 wild type overexpression constructs are around 2-fold above the endogenous EWS-FLI1 levels, it is possible that co-factors expressed at endogenous levels or interacting proteins are limiting further modulation. The knowledge of the EWS-FLI1 interactome is continuously expanding (28,29), however, it still remains unsolved which co-factors or interacting proteins are most important or influence the fusion protein in a context dependent matter. Nevertheless, longer induction of wild type or mutated ectopic EWS-FLI1 up to 96h induced cell death irrespective of the construct (data not shown).

Besides the majority of unmodified target genes, two other subgroups display a different behavior. 15 activated target genes are higher expressed upon stabilized EWS-FLI1 protein levels. This group includes protein-coding genes such as IGF1 (41) or LEMD1 and RNA-coding genes such as EWSAT1 (48). Interestingly, EWSAT1 was also observed to be elevated upon higher EWS-FLI1 expression in a different experimental setting (48) suggesting that our approach reflects a global pattern. Further, 29 activated target genes are less expressed upon stabilized EWS-FLI1. None of these turnover sensitive target genes have yet been characterized even though few interesting candidates as ALK or TP63 appeared.

Most interestingly, the activity of TFs can be influenced by their own turnover as shown for estrogen receptor α or c-MYC (58,59). It was recently shown that the proteasomal turnover of c-myc is required for full activity. Lysine mutated c-MYC failed to induce tumorigenesis as inhibitory complexes could not be removed during target gene activation (58) while other TFs are independent of ongoing degradation for their transcriptional activity (60). Surprisingly, EWS-FLI1 induced distinct behavior in three subgroups of target genes. Speculatively, the two small subgroups of EWS-FLI1 protein sensitive and turnover sensitive target genes might be of greater importance and their dynamics might trigger different response pathways in Ewing sarcoma

oncogenicity. Even though it is not yet clear how these subgroups are defined at their regulatory regions, possible driver target genes might be found in these two subgroups. Alternatively, since we see most target gene levels unaltered, oncogenic properties might depend also on additional mechanisms such as interacting proteins and/or influence on the splicing machinery.

Taken together, turnover regulation of the major oncogenic driver EWS-FLI1 represents an important regulatory mechanism in Ewing sarcoma pathogenesis. Interference with fusion protein degradation might not only be a novel therapeutic strategy in Ewing sarcoma treatment, but might also be applicable to other ETS based fusion proteins.

Experimental procedure

Cell lines and reagents- HEK293T cells were cultured in DMEM (Sigma Aldrich, Buchs, Switzerland) supplemented with 10% FBS (Sigma Aldrich), 2mmol/L glutamine (BioConcept, Allschwil, Switzerland) and 100U/ml penicillin/streptomycin (ThermoFisher Scientific AG, Reinach, Switzerland) at 37°C in 5% CO_2. Ewing sarcoma cell lines A673, SKNMC and TC71 were cultured in RPMI as described above. Additionally, dishes were pre-coated with 0.2% gelatine (Sigma Aldrich). All cell lines have been tested mycoplasma negative. The following reagents were used: Bortezomib (Selleckchem, Houston, TX, USA), Chloroquine, cycloheximide, DMSO, doxycycline, E64, leptomycin B (all Sigma Aldrich), LY294002, MG-132 (Millipore, Billerica, MA, USA) and 3-Methyladenine (ApexBio, Houston, TX, USA).

Plasmids and cloning- The coding sequence for human EWS-FLI1, FLI1 and EWS, were subcloned into the Not1 site of pCMV-3xflag vector (Sigma Aldrich). For Global Protein Stability, the coding sequences of DsRed-IRES-EGFP or d24-EGFP or d4-EGFP or d1-EGFP (addgene #41941, #41944, #41943, #41942) were cloned into the EcoR1 site of pR-EF1 (Cellecta Inc., Mountain View, CA, USA). All cDNAs (EWS-FLI1, FLI1, EWS and mutants) were inserted into the BSTB1 site at the 3' end of DsRed-IRES-EGFP.

3xflag-EWS-FLI1 cDNA was cloned into the Spe1-BamH1 sites of pInducer21 ORF (Addgene #46948). The pRSIT-U6Tet-shRNA-PGKTetRep-2A-GFP-2A-puro vector with shRNAs against EWS-FLI1 was purchased from Cellecta Inc. with the following target sequences: shEF#1 5' ATAGAGGTGGGAAGCTTATAA 3' (previously described in (31)) and shEF#2 5' CGTCATGTTCTGGTTTGAGAT 3' (designed against the C-terminal FLI1 part according to RefSeq# NM_002017.4).
Cloning was performed by In-Fusion cloning HD (Clontech Laboratories Inc., Mountain View, CA, USA) according to manufacturer's protocol. All mutations were introduced using site-directed mutagenesis. Detailed information of plasmids, cloning and mutagenesis primers can be found in supplementary table ST1. All clonings were verified by sequencing.

Transient transfection- For HEK293T cells, DMEM complete medium was mixed with PolyethylenimineMax (Polyscience, Cham, Switzerland) and plasmids for 15min and added to the cells for 48h. For Ewing sarcoma cells, JetPrime (Polyplus Transfections, Illkirch, France) reagent was used according to manufacturer's instruction in antibiotics-free RPMI medium.

Viral production and transduction- HEK293T cells were transfected with cDNA vectors and pMDL, pREV, and pVSV with JetPrime according to manufacturer's instruction in antibiotics-free DMEM medium. Medium was replaced 24h after transfection and virus was harvested after additional 48h. Viral supernatant was cleared by centrifugation, filtered and concentrated if necessary (Amicon® Ultra 15 mL, Millipore). Ewing sarcoma cells were infected with the viral supernatant supplemented with 10µg/ml polybrene (Sigma Aldrich) for 16-18h.

Global Protein Stability Assay and flow cytometry- Lentivirus from HEK293T transfected with pR-EF1-DsRed-IRES-d1/d4/d24/EGFP-cDNA, pMDL, pREV and pVSV was used to transduce HEK293T or Ewing sarcoma cells as described to about 5-10% of DsRed positive cells. Cells were harvested 72h after transduction, incubated with cycloheximide, leptomycin B or MG-132 for 4-8h as indicated and analyzed by FACS. Cells were resuspended in PBS and filtered

using a 40µm cell strainer (BD Becton, Dickinson and Company, Franklin Lakes, NJ, USA). FACS analysis was performed on a FACS Canto™ II Cytometry system (BD) and data were analyzed by FlowJo software (Treestar Inc., Ashland, OR, USA). At least 50000 cells were recorded for each experiment. Cell sorting was done with a FACS AriaTM III Cytometry system (BD).

Cell lysis, western blotting and antibodies- Cells were harvested and lysed in standard lysis buffer (50mM Tris/HCl, 150mM NaCl, 50mM NaF, 5mM $Na_4P_2O_7$, 1mM Na_3VO_4 and 10mM ß-glycerolphosphate, 1% TritonX with protease inhibitor cocktail, Complete Mini® (Sigma Aldrich)). Lysates were sonicated and cleared by centrifugation. Proteins were separated by 4%-12% or 10% BisTris NuPAGE pre-cast gels (ThermoFisher Scientific AG) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% milk powder in 0.2% PBS-Tween and primary antibodies were incubated over night at 4°C followed by 2h of HRP-linked secondary antibody at room temperature. Proteins were detected by chemiluminescence using Amersham ECL detection reagent (GE Healthcare, Glattbrugg, Switzerland) or SuperSignal™ Western blotting reagent (ThermoFisher Scientific AG). Quantification of blots was performed by using ImageJ (version 1.46r). The following commercial antibodies were used: anti-Actin (dilution 1:1000, Cell Signaling), anti-Flag (clone M2, 1:1000, Sigma Aldrich), anti-FLI1 (1:1000, MyBiosource LLC, San Diego, CA, USA), anti-GAPDH (1:1000, Cell Signaling), anti-HA (1:1000, Millipore), anti-LAMP1 (1:500, DSHB, Iowa City, Iowa, USA), anti-p27 (1:1000, Cell Signaling and 1:200 ThermoFisher Scientific AG) and anti-Tubulin (1:40000 Sigma Aldrich).

Immunoprecipitation of ubiquitinated proteins- Cells were transfected for 48h and treated with 10µM MG-132 prior to lysis in ubiquitin lysis buffer (2% SDS, 150mM NaCl, 10mM Tris/HCl, 2mM Na_3VO_4, 50mM NaF with Complete Mini Protease inhibitor cocktail), boiled for 10min and sonicated. Lysates were diluted 1:10 in dilution buffer (150mM NaCl, 10mM Tris/HCl, 2mM EDTA, 1% Triton X), incubated for 30min at 4°C and cleared by centrifugation for 30min at maximum speed. Immunoprecipitation was performed using anti-Flag antibody (Sigma Aldrich) coupled to Dynabeads ProteinG (ThermoFisher Scientific AG). Lysates were incubated for 1h at 4°C, washed three times, eluted at room temperature using 3xflag peptide (Sigma Aldrich) and prepared for western blot analysis. Extended description for detection of EWS-FLI1 ubiquitination sites by mass spectrometry can be found in supplementary S1.

Immunofluorescence and microscopy- A673 cells were seeded on cover slides for 24h and transiently transfected with flag tagged plasmids for additional 48h. After fixing with 4% PFA (Carl Roth, Arlesheim, Switzerland), cells were permeabilized and stained with anti-Flag antibody (1:300, Sigma Aldrich) in 4% horse serum (Sigma Aldrich) and 0.1% PBS-TritonX over night. Fluorescent secondary antibody (Alexa-488 anti-mouse, Sigma Aldrich) in PBS with 4% horse serum was applied for 1h. Cover slides were fixed on objective glass with DAPI Vectashield® mounting medium (Vector laboratories Inc., Burlingame, CA, USA) and analyzed by an Axioskop 2 MOT Plus (Carl Zeiss Microscopy LLC, Thornwood, NY, USA).

Quantitative PCR and microarray analysis- Total RNA was extracted from Ewing sarcoma cells using Qiagen RNeasy Kit (Qiagen Instruments AG, Hombrechtikon, Switzerland). cDNA synthesis was carried out using High-Capacity Reverse Transcription Kit (Applied Biosystems by ThermoFisher Scientific AG). Quantitative PCR was performed using TaqMan gene expression master mix (ThermoFisher Scientific AG) and assays on demand (Applied Biosystems by ThermoFisher Scientific AG). A complete list can be found in supplementary table ST1. Replicate values were pooled and represented as geometric mean values with a 95% confidence interval. Microarray expression analysis with total RNA was performed using GeneChip® Human Gene 2.0 ST Array (Atlas Biolabs, Berlin, Germany). Data are accessible under GSE81018.

Microarray data analysis- The raw microarray data were processed and normalized using the RMA algorithm (61). Differential expression was computed using the Bioconductor package limma providing a moderated t-test that is adapted to low number of replicates (62).

Differential expression results were filtered based on fold-change and p-values. All computations were done using R/Bioconductor. Extended description for target gene grouping can be found in supplementary S2.

FOOTNOTES

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Author's contributions: MG, BS, FN designed the study and wrote the paper. MG and FP designed, performed and analyzed the experiments. LL performed and analyzed the mass spectrometry experiments. LH provided help with flow cytometry and sorted stable cell lines. All authors reviewed the results and approved the final version of the manuscript.

References

1. Hollenhorst, P. C., McIntosh, L. P., and Graves, B. J. (2011) Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu Rev Biochem* **80**, 437-471
2. Laudet, V., Hanni, C., Stehelin, D., and Duterque-Coquillaud, M. (1999) Molecular phylogeny of the ETS gene family. *Oncogene* **18**, 1351-1359
3. Wasylyk, B., Hahn, S. L., and Giovane, A. (1993) The Ets family of transcription factors. *Eur J Biochem* **211**, 7-18
4. Li, R., Pei, H., and Watson, D. K. (2000) Regulation of Ets function by protein - protein interactions. *Oncogene* **19**, 6514-6523
5. Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D., and Graves, B. J. (1992) Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. *Genes Dev* **6**, 975-990
6. Lelievre, E., Lionneton, F., Soncin, F., and Vandenbunder, B. (2001) The Ets family contains transcriptional activators and repressors involved in angiogenesis. *Int J Biochem Cell Biol* **33**, 391-407
7. Sharrocks, A. D. (2001) The ETS-domain transcription factor family. *Nat Rev Mol Cell Biol* **2**, 827-837
8. Davidson, B., Reich, R., Goldberg, I., Gotlieb, W. H., Kopolovic, J., Berner, A., Ben-Baruch, G., Bryne, M., and Nesland, J. M. (2001) Ets-1 messenger RNA expression is a novel marker of poor survival in ovarian carcinoma. *Clin Cancer Res* **7**, 551-557
9. Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D., and Gilliland, D. G. (1995) Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A* **92**, 4917-4921
10. Petrovics, G., Liu, A., Shaheduzzaman, S., Furusato, B., Sun, C., Chen, Y., Nau, M., Ravindranath, L., Chen, Y., Dobi, A., Srikantan, V., Sesterhenn, I. A., McLeod, D. G., Vahey, M., Moul, J. W., and Srivastava, S. (2005) Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. *Oncogene* **24**, 3847-3852
11. Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., Mehra, R., Sun, X. W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., Lee, C., Montie, J. E., Shah, R. B., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* **310**, 644-648
12. Kumar-Sinha, C., Tomlins, S. A., and Chinnaiyan, A. M. (2008) Recurrent gene fusions in prostate cancer. *Nat Rev Cancer* **8**, 497-511
13. Seth, A., and Watson, D. K. (2005) ETS transcription factors and their emerging roles in human cancer. *Eur J Cancer* **41**, 2462-2478
14. Mertens, F., Antonescu, C. R., Hohenberger, P., Ladanyi, M., Modena, P., D'Incalci, M., Casali, P. G., Aglietta, M., and Alvegard, T. (2009) Translocation-related sarcomas. *Semin Oncol* **36**, 312-323
15. Mitelman, F., Johansson, B., and Mertens, F. (2007) The impact of translocations and gene fusions on cancer causation. *Nat Rev Cancer* **7**, 233-245
16. Hagenbuchner, J., and Ausserlechner, M. J. (2015) Targeting transcription factors by small compounds-Current strategies and future implications. *Biochem Pharmacol*
17. Bernstein, M., Kovar, H., Paulussen, M., Randall, R. L., Schuck, A., Teot, L. A., and Juergens, H. (2006) Ewing's sarcoma family of tumors: Current management. *Oncologist* **11**, 503-519
18. Esiashvili, N., Goodman, M., and Marcus, R. B., Jr. (2008) Changes in incidence and survival of Ewing sarcoma patients over the past 3 decades: Surveillance Epidemiology and End Results data. *J Pediatr Hematol Oncol* **30**, 425-430

19. Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., and et al. (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature* **359**, 162-165
20. May, W. A., Gishizky, M. L., Lessnick, S. L., Lunsford, L. B., Lewis, B. C., Delattre, O., Zucman, J., Thomas, G., and Denny, C. T. (1993) Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation. *Proc Natl Acad Sci U S A* **90**, 5752-5756
21. May, W. A., Lessnick, S. L., Braun, B. S., Klemsz, M., Lewis, B. C., Lunsford, L. B., Hromas, R., and Denny, C. T. (1993) The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. *Mol Cell Biol* **13**, 7393-7398
22. Bailly, R. A., Bosselut, R., Zucman, J., Cormier, F., Delattre, O., Roussel, M., Thomas, G., and Ghysdael, J. (1994) DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. *Mol Cell Biol* **14**, 3230-3241
23. Braun, B. S., Frieden, R., Lessnick, S. L., May, W. A., and Denny, C. T. (1995) Identification of target genes for the Ewing's sarcoma EWS/FLI fusion protein by representational difference analysis. *Mol Cell Biol* **15**, 4623-4630
24. Kovar, H., Aryee, D. N., Jug, G., Henockl, C., Schemper, M., Delattre, O., Thomas, G., and Gadner, H. (1996) EWS/FLI-1 antagonists induce growth inhibition of Ewing tumor cells in vitro. *Cell Growth Differ* **7**, 429-437
25. Tanaka, K., Iwakuma, T., Harimaya, K., Sato, H., and Iwamoto, Y. (1997) EWS-Fli1 antisense oligodeoxynucleotide inhibits proliferation of human Ewing's sarcoma and primitive neuroectodermal tumor cells. *J Clin Invest* **99**, 239-247
26. Toretsky, J. A., Connell, Y., Neckers, L., and Bhat, N. K. (1997) Inhibition of EWS-FLI-1 fusion protein with antisense oligodeoxynucleotides. *J Neurooncol* **31**, 9-16
27. Petermann, R., Mossier, B. M., Aryee, D. N., Khazak, V., Golemis, E. A., and Kovar, H. (1998) Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II. *Oncogene* **17**, 603-610
28. Selvanathan, S. P., Graham, G. T., Erkizan, H. V., Dirksen, U., Natarajan, T. G., Dakic, A., Yu, S., Liu, X., Paulsen, M. T., Ljungman, M. E., Wu, C. H., Lawlor, E. R., Uren, A., and Toretsky, J. A. (2015) Oncogenic fusion protein EWS-FLI1 is a network hub that regulates alternative splicing. *Proc Natl Acad Sci U S A* **112**, E1307-1316
29. Toretsky, J. A., Erkizan, V., Levenson, A., Abaan, O. D., Parvin, J. D., Cripe, T. P., Rice, A. M., Lee, S. B., and Uren, A. (2006) Oncoprotein EWS-FLI1 activity is enhanced by RNA helicase A. *Cancer Res* **66**, 5574-5581
30. Kinsey, M., Smith, R., and Lessnick, S. L. (2006) NR0B1 is required for the oncogenic phenotype mediated by EWS/FLI in Ewing's sarcoma. *Mol Cancer Res* **4**, 851-859
31. Smith, R., Owen, L. A., Trem, D. J., Wong, J. S., Whangbo, J. S., Golub, T. R., and Lessnick, S. L. (2006) Expression profiling of EWS/FLI identifies NKX2.2 as a critical target gene in Ewing's sarcoma. *Cancer Cell* **9**, 405-416
32. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu Rev Biochem* **67**, 425-479
33. An, J., Ren, S., Murphy, S. J., Dalangood, S., Chang, C., Pang, X., Cui, Y., Wang, L., Pan, Y., Zhang, X., Zhu, Y., Wang, C., Halling, G. C., Cheng, L., Sukov, W. R., Karnes, R. J., Vasmatzis, G., Zhang, Q., Zhang, J., Cheville, J. C., Yan, J., Sun, Y., and Huang, H. (2015) Truncated ERG Oncoproteins from TMPRSS2-ERG Fusions Are Resistant to SPOP-Mediated Proteasome Degradation. *Mol Cell* **59**, 904-916
34. Gan, W., Dai, X., Lunardi, A., Li, Z., Inuzuka, H., Liu, P., Varmeh, S., Zhang, J., Cheng, L., Sun, Y., Asara, J. M., Beck, A. H., Huang, J., Pandolfi, P. P., and Wei, W. (2015) SPOP Promotes

Ubiquitination and Degradation of the ERG Oncoprotein to Suppress Prostate Cancer Progression. *Mol Cell* **59**, 917-930

35. Ji, Z., Degerny, C., Vintonenko, N., Deheuninck, J., Foveau, B., Leroy, C., Coll, J., Tulasne, D., Baert, J. L., and Fafeur, V. (2007) Regulation of the Ets-1 transcription factor by sumoylation and ubiquitinylation. *Oncogene* **26**, 395-406
36. Vitari, A. C., Leong, K. G., Newton, K., Yee, C., O'Rourke, K., Liu, J., Phu, L., Vij, R., Ferrando, R., Couto, S. S., Mohan, S., Pandita, A., Hongo, J. A., Arnott, D., Wertz, I. E., Gao, W. Q., French, D. M., and Dixit, V. M. (2011) COP1 is a tumour suppressor that causes degradation of ETS transcription factors. *Nature* **474**, 403-406
37. Elzi, D. J., Song, M., Hakala, K., Weintraub, S. T., and Shiio, Y. (2014) Proteomic Analysis of the EWS-Fli-1 Interactome Reveals the Role of the Lysosome in EWS-Fli-1 Turnover. *J Proteome Res* **13**, 3783-3791
38. Yen, H. C., Xu, Q., Chou, D. M., Zhao, Z., and Elledge, S. J. (2008) Global protein stability profiling in mammalian cells. *Science* **322**, 918-923
39. Liang, H., Mao, X., Olejniczak, E. T., Nettesheim, D. G., Yu, L., Meadows, R. P., Thompson, C. B., and Fesik, S. W. (1994) Solution structure of the ets domain of Fli-1 when bound to DNA. *Nat Struct Biol* **1**, 871-875
40. Welford, S. M., Hebert, S. P., Deneen, B., Arvand, A., and Denny, C. T. (2001) DNA binding domain-independent pathways are involved in EWS/FLI1-mediated oncogenesis. *J Biol Chem* **276**, 41977-41984
41. Riggi, N., Suva, M. L., Suva, D., Cironi, L., Provero, P., Tercier, S., Joseph, J. M., Stehle, J. C., Baumer, K., Kindler, V., and Stamenkovic, I. (2008) EWS-FLI-1 expression triggers a Ewing's sarcoma initiation program in primary human mesenchymal stem cells. *Cancer Res* **68**, 2176-2185
42. Kinsey, M., Smith, R., Iyer, A. K., McCabe, E. R., and Lessnick, S. L. (2009) EWS/FLI and its downstream target NR0B1 interact directly to modulate transcription and oncogenesis in Ewing's sarcoma. *Cancer Res* **69**, 9047-9055
43. Tirado, O. M., Mateo-Lozano, S., Villar, J., Dettin, L. E., Llort, A., Gallego, S., Ban, J., Kovar, H., and Notario, V. (2006) Caveolin-1 (CAV1) is a target of EWS/FLI-1 and a key determinant of the oncogenic phenotype and tumorigenicity of Ewing's sarcoma cells. *Cancer Res* **66**, 9937-9947
44. Boro, A., Pretre, K., Rechfeld, F., Thalhammer, V., Oesch, S., Wachtel, M., Schafer, B. W., and Niggli, F. K. (2012) Small-molecule screen identifies modulators of EWS/FLI1 target gene expression and cell survival in Ewing's sarcoma. *Int J Cancer* **131**, 2153-2164
45. Prieur, A., Tirode, F., Cohen, P., and Delattre, O. (2004) EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. *Mol Cell Biol* **24**, 7275-7283
46. Sankar, S., Bell, R., Stephens, B., Zhuo, R., Sharma, S., Bearss, D. J., and Lessnick, S. L. (2013) Mechanism and relevance of EWS/FLI-mediated transcriptional repression in Ewing sarcoma. *Oncogene* **32**, 5089-5100
47. Matsunobu, T., Tanaka, K., Nakamura, T., Nakatani, F., Sakimura, R., Hanada, M., Li, X., Okada, T., Oda, Y., Tsuneyoshi, M., and Iwamoto, Y. (2006) The possible role of EWS-Fli1 in evasion of senescence in Ewing family tumors. *Cancer Res* **66**, 803-811
48. Marques Howarth, M., Simpson, D., Ngok, S. P., Nieves, B., Chen, R., Siprashvili, Z., Vaka, D., Breese, M. R., Crompton, B. D., Alexe, G., Hawkins, D. S., Jacobson, D., Brunner, A. L., West, R., Mora, J., Stegmaier, K., Khavari, P., and Sweet-Cordero, E. A. (2014) Long noncoding RNA EWSAT1-mediated gene repression facilitates Ewing sarcoma oncogenesis. *J Clin Invest* **124**, 5275-5290
49. Williamson, A., Werner, A., and Rape, M. (2013) The Colossus of ubiquitylation: decrypting a cellular code. *Mol Cell* **49**, 591-600

50. Emanuele, M. J., Elia, A. E., Xu, Q., Thoma, C. R., Izhar, L., Leng, Y., Guo, A., Chen, Y. N., Rush, J., Hsu, P. W., Yen, H. C., and Elledge, S. J. (2011) Global identification of modular cullin-RING ligase substrates. *Cell* **147**, 459-474
51. Yen, H. C., and Elledge, S. J. (2008) Identification of SCF ubiquitin ligase substrates by global protein stability profiling. *Science* **322**, 923-929
52. Kodadek, T., Sikder, D., and Nalley, K. (2006) Keeping transcriptional activators under control. *Cell* **127**, 261-264
53. Muratani, M., and Tansey, W. P. (2003) How the ubiquitin-proteasome system controls transcription. *Nat Rev Mol Cell Biol* **4**, 192-201
54. Bachmaier, R., Aryee, D. N., Jug, G., Kauer, M., Kreppel, M., Lee, K. A., and Kovar, H. (2009) O-GlcNAcylation is involved in the transcriptional activity of EWS-FLI1 in Ewing's sarcoma. *Oncogene* **28**, 1280-1284
55. Schlottmann, S., Erkizan, H. V., Barber-Rotenberg, J. S., Knights, C., Cheema, A., Uren, A., Avantaggiati, M. L., and Toretsky, J. A. (2012) Acetylation Increases EWS-FLI1 DNA Binding and Transcriptional Activity. *Front Oncol* **2**, 107
56. Klevernic, I. V., Morton, S., Davis, R. J., and Cohen, P. (2009) Phosphorylation of Ewing's sarcoma protein (EWS) and EWS-Fli1 in response to DNA damage. *Biochem J* **418**, 625-634
57. Grunewald, T. G., Diebold, I., Esposito, I., Plehm, S., Hauer, K., Thiel, U., da Silva-Buttkus, P., Neff, F., Unland, R., Muller-Tidow, C., Zobywalski, C., Lohrig, K., Lewandrowski, U., Sickmann, A., Prazeres da Costa, O., Gorlach, A., Cossarizza, A., Butt, E., Richter, G. H., and Burdach, S. (2012) STEAP1 is associated with the invasive and oxidative stress phenotype of Ewing tumors. *Mol Cancer Res* **10**, 52-65
58. Jaenicke, L. A., von Eyss, B., Carstensen, A., Wolf, E., Xu, W., Greifenberg, A. K., Geyer, M., Eilers, M., and Popov, N. (2016) Ubiquitin-Dependent Turnover of MYC Antagonizes MYC/PAF1C Complex Accumulation to Drive Transcriptional Elongation. *Mol Cell* **61**, 54-67
59. Reid, G., Hubner, M. R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., Ellenberg, J., and Gannon, F. (2003) Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell* **11**, 695-707
60. Yao, J., Munson, K. M., Webb, W. W., and Lis, J. T. (2006) Dynamics of heat shock factor association with native gene loci in living cells. *Nature* **442**, 1050-1053
61. Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., and Speed, T. P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249-264
62. Gentleman, R. (2005) *Bioinformatics and computational biology solutions using R and Bioconductor*, Springer Science+Business Media, New York

FIGURE LEGENDS

Figure 1: EWS-FLI1 protein turnover is proteasome dependent. (A) Western blot analysis of EWS-FLI1 protein levels. A673 cells were treated with 20µM of indicated compounds for 8h, EWS-FLI1 protein levels were detected with anti-FLI1 antibody. Quantification represents ratio of FLI1 over GAPDH compared to DMSO control. (B) EWS-FLI1 turnover in various Ewing sarcoma cell lines. A673, SKNMC and TC71 cells were treated with 20µM MG-132 or chloroquine (CQ) for 10h (A673), 8h (SKNMC) and 9h (TC71), and immunoblotted with anti-FLI1 antibody. Quantification represents ratio of FLI1 over tubulin compared to DMSO control. (C) EWS-FLI1 stabilizes in a time-dependent manner. Western blot of A673 cells treated with 10µM MG-132 for indicated time points. Three independent experiments were quantified and are represented in the scatter blot with n=3 (2h-8h) or n=5 (DMSO), error bars as SD. (D) Half-life of endogenous EWS-FLI1 protein. Western blot of A673 cells treated with 20µg/ml CHX for indicated hours. Quantification of three independent experiments with n=3 (2h-8h) or n=5 (DMSO), error bars as SD. (E) EWS-FLI1 is ubiquitinated. 3xflag-EWS-FLI1 and HA-ubiquitin were co-expressed for 48h in HEK293T cells and incubated with 10µM MG-132 for indicated hours. After immunoprecipitation of EWS-FLI1 with anti-Flag, ubiquitination was visualized by anti-HA antibody. (F) EWS-FLI1 ubiquitination consists of K48-linked ubiquitin chains. 3xflag-EWS-FLI1 and wild-type or mutant HA-ubiquitins were co-expressed for 48h in HEK293T cells, immunoprecipitated and ubiquitination was visualized by anti-HA antibody.

Figure 2: The EWS-FLI1 protein displays a high turnover. (A) Scheme illustrating GPS approach in which a reporter construct of DsRed-IRES-EGFP is fused at the C-terminus of EGFP to a protein of interest. The EGFP/DsRed ratio is determined by FACS and represents a measure for protein stability. (B) EWS-FLI1 turnover measured by GPS. HEK293T were transduced for 72h with a reporter construct fused to EWS-FLI1 or degron (d) motifs with half lives of 1h (d1h), 4h (d4h) and 24h (d24h) and analyzed by FACS. The reporter construct fused to EWS-FLI1 was additionally incubated with 20µM MG-132 or 20µg/ml CHX for 8h or (C) with 50nM Leptomycin B (LMB) for 4h and 8h (dotted line). (D) EWS-FLI1 stability in Ewing cell line A673. GPS analysis of reporter constructs fused to EWS-FLI1 or standard degron motifs 72h after transduction. (E) A673 cells stably transfected with reporter-EWS-FLI1 construct were sorted and incubated with DMSO, 20µM MG-132 or 20µg/ml CHX for 8h.

Figure 3: Mass spectrometry identifies K380 residue as the main ubiquitination site. (A) Two lysine residues were identified to be ubiquitinated by mass spectrometry. Peptide spectra with glycine-glycine shift for residue K380 (peptide spectra for the K298 site in supplementary S1A). (B) Mutation of lysine residue K380 prevents EWS-FLI1 ubiquitination. 3xflag-EWS-FLI1, K298R and K380R mutants were co-expressed with HA-ubiquitin for 48h in A673 cells, purified ubiquitinated EWS-FLI1 was analyzed using anti-HA antibody by western blotting. (C) Mutated K380 residue stabilizes EWS-FLI1 protein. 3xflag-EWS-FLI1, single and double mutants were transiently overexpressed for 48h in A673 and SKNMC cells and analyzed with an anti-Flag antibody by western blot. (D) Scatter blot for quantification for n=3 independent experiments, error bars as SD. (E) EWS-FLI1 mutant show nuclear localization. 3xflag-EWS-FLI1 and K380R single mutant were transiently expressed for 48h in A673 cells. Cells were fixed, stained with anti-Flag antibody and used for immunofluorescence (40x magnification). (F) Mutation of K380 stabilizes EWS-FLI1 posttranslationally. HEK293T cells were transduced for 72h with reporter constructs of EWS-FLI1, mutants K298R and K380R and standards. EGFP/DsRed ratios were analyzed by FACS, (G) after additional incubation with DMSO or 20µM MG-132 for 8h.

Figure 4: Regulation of proteasomal turnover is conserved between EWS-FLI1 and FLI1. (A) EWS-FLI1 displays fastest turnover. GPS analysis of EWS-FLI1, EWSR1 and FLI1 reporter constructs and standards transduced into HEK293T cells and analyzed after 72h by FACS. (B) Full length

proteins EWSR1 and FLI1 were additionally treated with 20µM MG-132 or 20µg/ml CHX for 8h. (C) Immunoprecipitation of EWS-FLI1, EWSR1 and FLI1 proteins. 3xflag tagged versions were co-expressed with HA-ubiquitin for 48h and stabilized for additional 5h with 20µM MG-132. After immunoprecipitation of tag proteins, ubiquitin pattern was analyzed by anti-HA antibody. (D) Mutation of conserved residue K334R stabilizes FLI1 in GPS analysis. HEK293T cells were transduced for 72h with reporter constructs of FLI1, mutants K172R, K252R, K334R and standards. EGFP/DsRed ratio was analyzed by FACS. (E) Turnover dependent lysine residue is conserved within most ETS family members. ClustalW alignment of conserved ETS domain sequence, K334/K380 residue is marked in red.

Figure 5: Generation of exchange Ewing cell lines. (A) EGFP (selection marker) sorting of pInducer21 vector transduced cells. A673 cells with 3xflag, 3xflag-EWS-FLI1 or 3xflag-K380R mutant were sorted for same low EGFP expression. After sorting, all cell pools were analyzed by FACS for EGFP and plotted for corresponding mean fluorescent intensity (MFI). (B) ShEF sequences shRNA against the 3' UTR of EWS-FLI1 are sufficient to downregulate EWS-FLI1 protein. Western blot of A673 cells with shEF constructs #1 and #2 after incubation with 0.1ng/µl doxycycline for 48h. (C) Inducible EWS-FLI1 exchange cell lines. Double transduced A673 cells were incubated with 0.1ng/µl doxycycline for 48h. Endogenous and exogenous EWS-FLI1 protein levels were analyzed by western blotting using anti-FLI1 antibody, (D) and mRNA levels were analyzed by quantitative RT-PCR, with n=4 and error bars represent 95% confidence intervals.

Figure 6: Differential regulation of target gene expression by EWS-FLI1 protein levels. (A) Analysis of microarray expression data of A673 exchange cell lines reveals three subgroups of target gene regulation. Pie chart distribution for EWS-FLI1 activated (upper) and repressed (lower) target genes and their differential modulation of stabilized over wild type EWS-FLI1. (B-C) Heat map of activated-rescued (B) and repressed-rescued (C) target gene signature. The comparison of stabilized over wild type EWS-FLI1 target genes resembled in three distinct groups. (D-E) Validation of selected activated (D) or repressed (E) target genes by quantitative RT-PCR based on RNA extracted from shEF#2 exchange cell lines, with n=4 and error bars represent 95% confidence intervals.

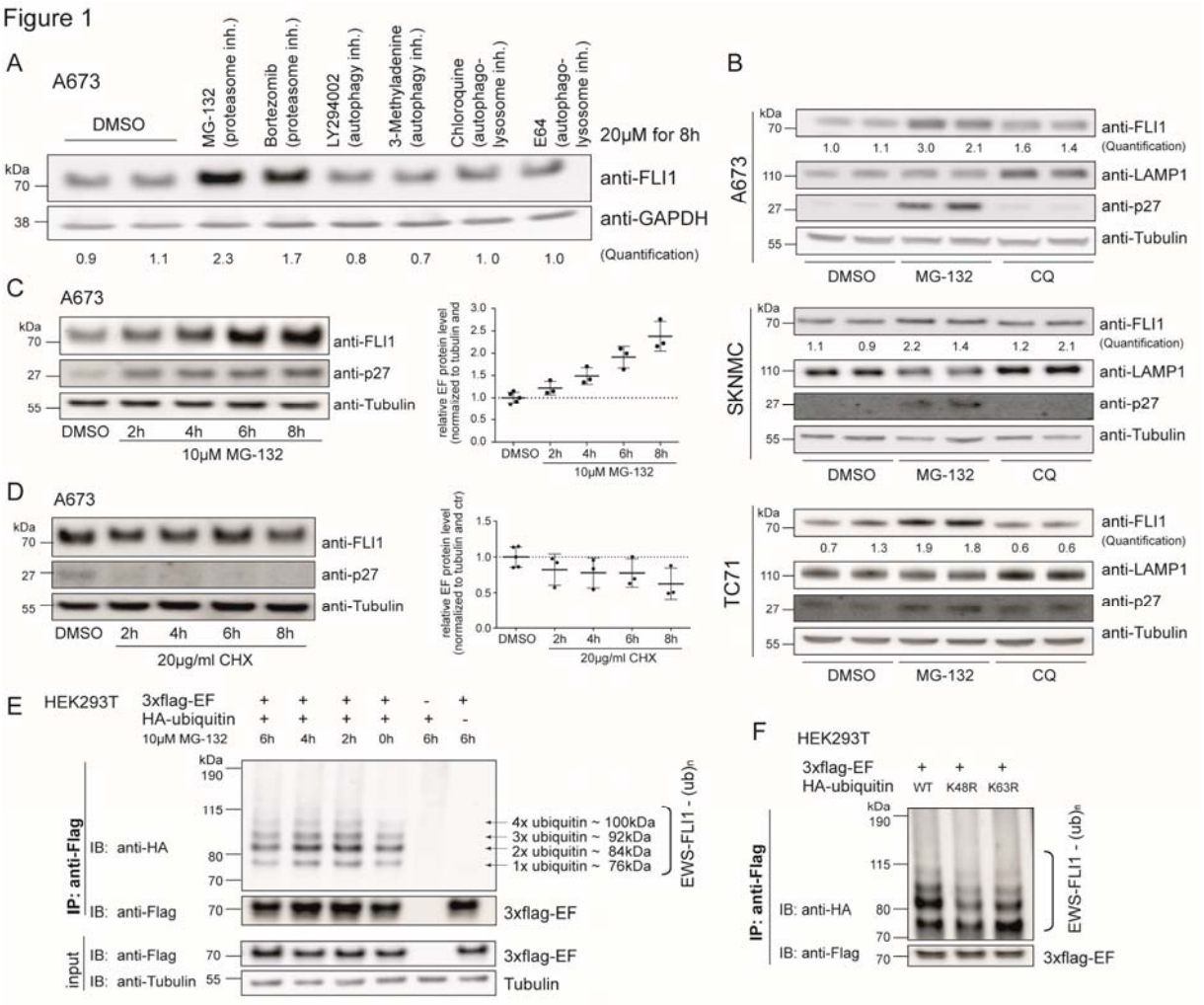


Figure 2

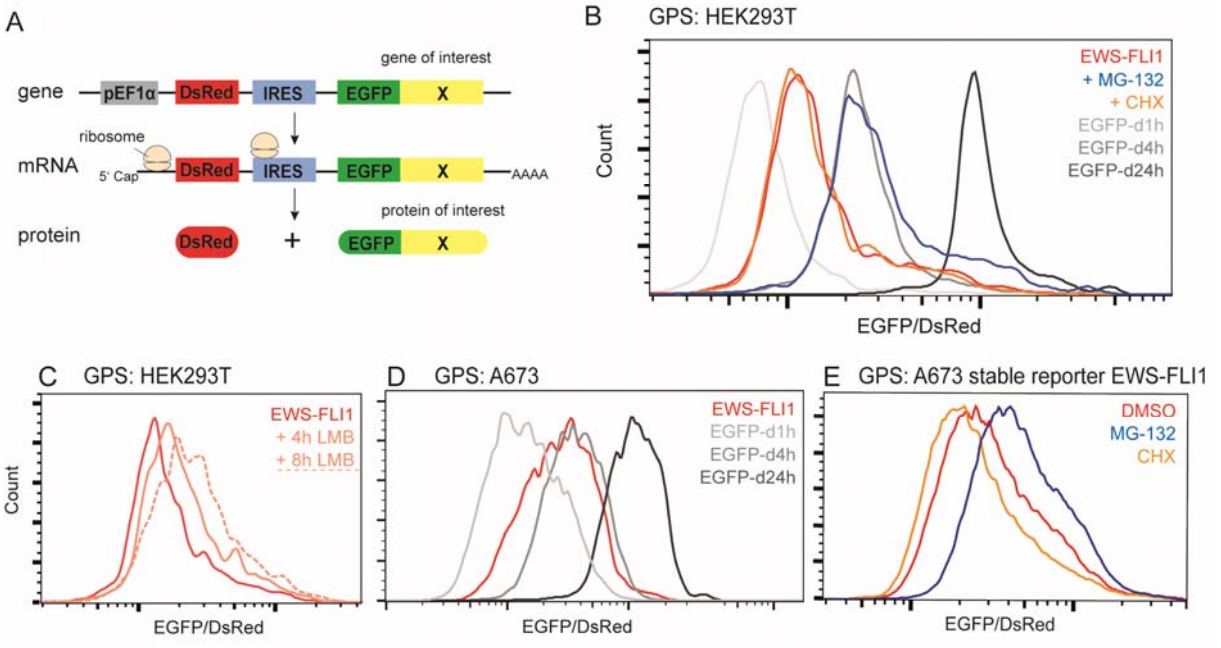


Figure 3

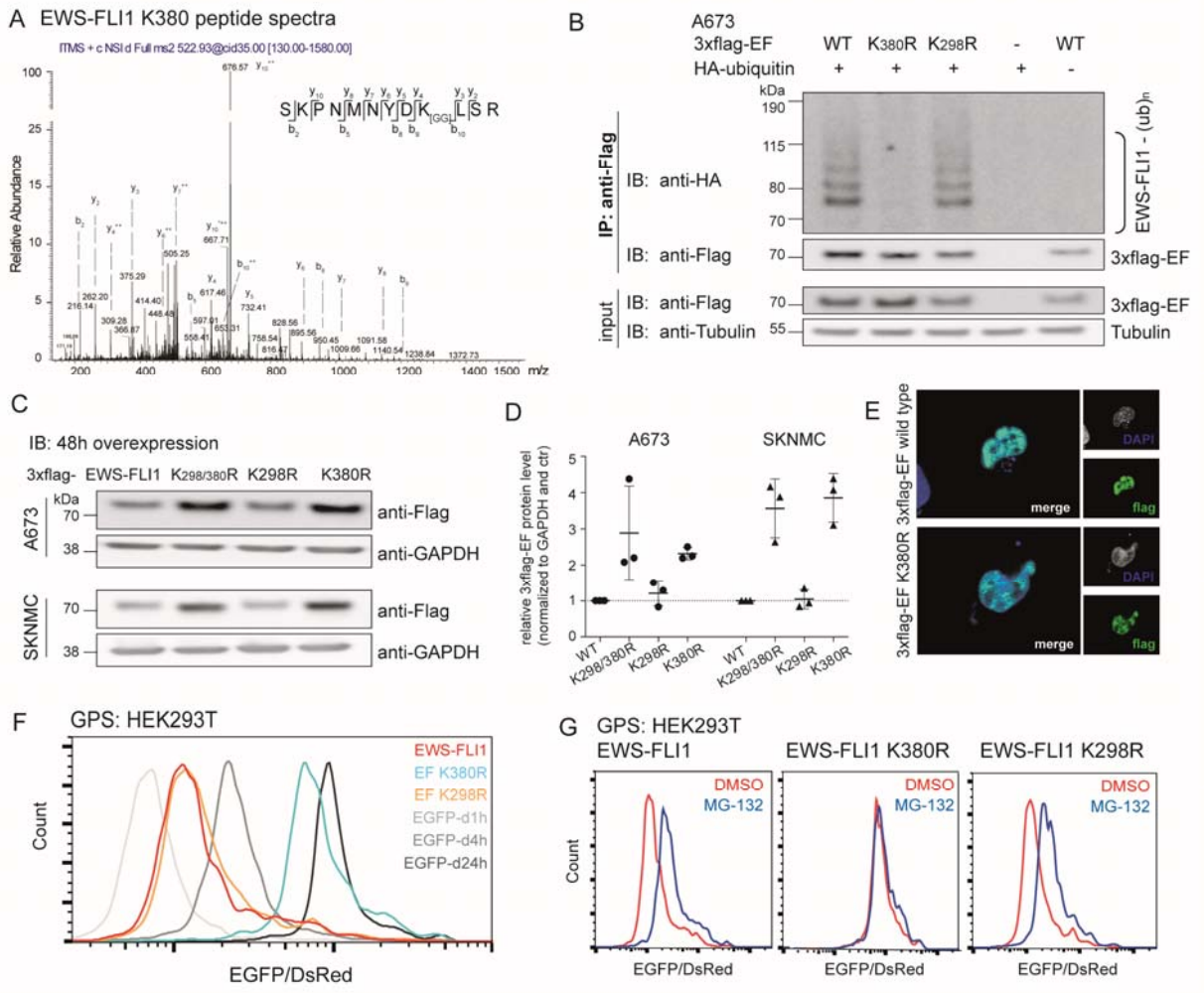
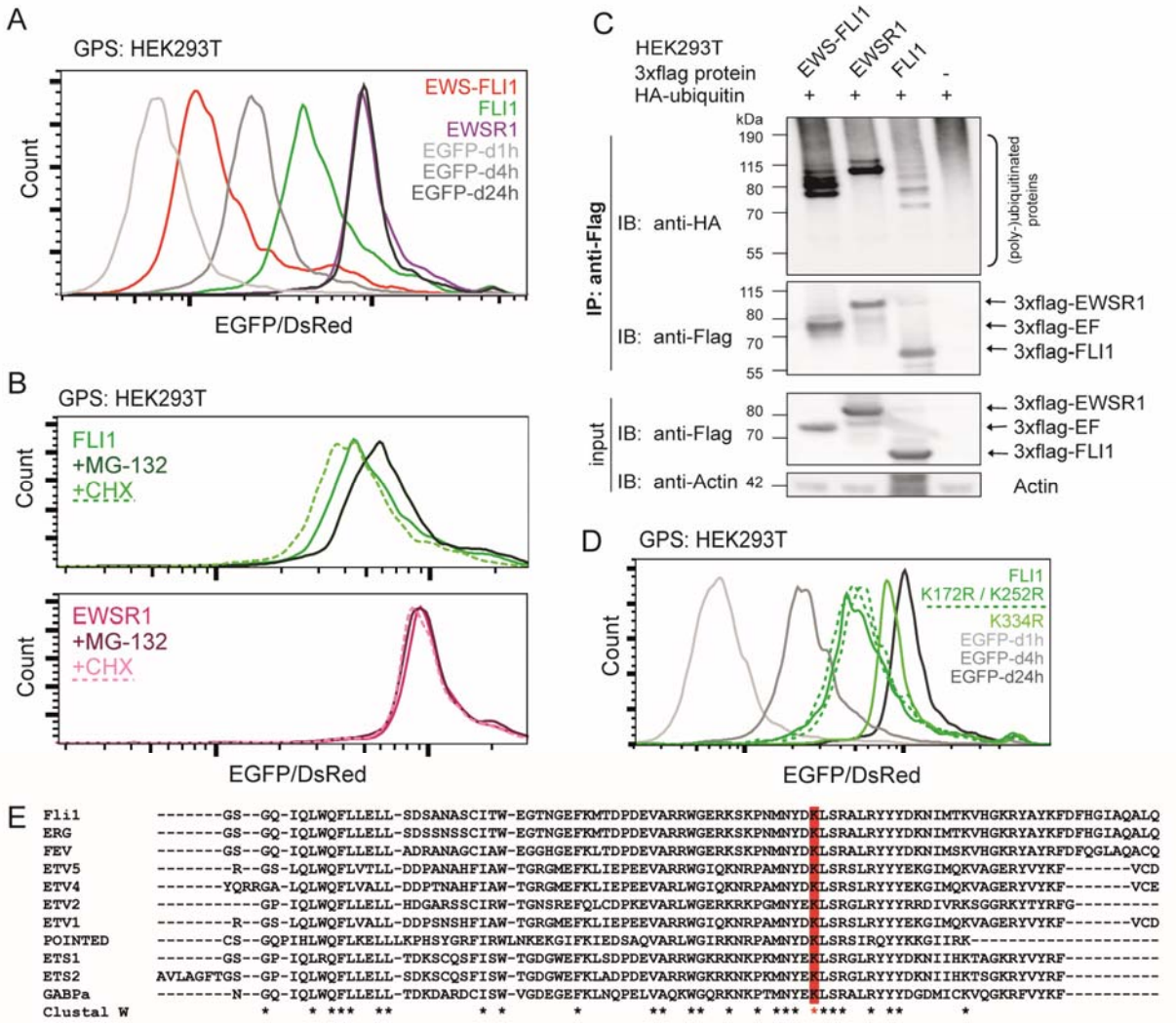


Figure 4



E

```
Fli1        -------GS--GQ-IQLWQFLLELL-SDSANASCITW-EGTNGEFKMTDPDEVARRWGERKSKPNMNYDKLSRALRYYYDKNIMTKVHGKRYAYKFDFHGIAQALQ
ERG         -------GS--GQ-IQLWQFLLELL-SDSSNSSCITW-EGTNGEFKMTDPDEVARRWGERKSKPNMNYDKLSRALRYYYDKNIMTKVHGKRYAYKFDFHGIAQALQ
FEV         -------GS--GQ-IQLWQFLLELL-ADRANAGCIAW-EGGHGEFKLTDPDEVARRWGERKSKPNMNYDKLSRALRYYYDKNIMSKVHGKRYAYRFDFQGLAQACQ
ETV5        --------R--GS-LQLWQFLVTLL-DDPANAHFIAW-TGRGMEFKLIEPEEVARRWGIQKNRPAMNYDKLSRSLRYYYEKGIMQKVAGERYVYKF-------VCD
ETV4        -------YQRRGA-LQLWQFLVALL-DDPTNAHFIAW-TGRGMEFKLIEPEEVARLWGIQKNRPAMNYDKLSRSLRYYYEKGIMQKVAGERYVYKF-------VCE
ETV2        -----------GP-IQLWQFLLELL-HDGARSSCIRW-TGNSREFQLCDPKEVARLWGERKRKPGMNYEKLSRGLRYYYRRDIVRKSGGRKYTYRFG---------
ETV1        --------R--GS-LQLWQFLVALL-DDPSNSHFIAW-TGRGMEFKLIEPEEVARRWGIQKNRPAMNYDKLSRSLRYYYEKGIMQKVAGERYVYKF-------VCD
POINTED     -------CS--GQPIHLWQFLKELLLKPHSYGRFIRWLNKEKGIFKIEDSAQVARLWGIRKNRPAMNYDKLSRSIRQYYKKGIIRK--------------------
ETS1        -------GS--GP-IQLRQFLLELL-TDKSCQSFISW-TGDGWEFKLSDPDEVARRWGKRKNKPKMNYEKLSRGLRYYYDKNIIHKTAGKRYVYRF----------
ETS2        AVLAGFTGS--GP-IQLWQFLLELL-SDKSCQSFISW-TGDGWEFKLADPDEVARRWGKRKNKPKMNYEKLSRGLRYYYDKNIIHKTSGKRYVYRF----------
GABPa       --------N--GQ-IQLWQFLLELL-TDKDARDCISW-VGDEGEFKLNQPELVAQKWGQRKNKPTMNYEKLSRALRYYYDGDMICKVQGKRFVYKF----------
Clustal W               *    * ***  **         * *       *       **  **  *  * *** ****  * **      *
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Figure 5

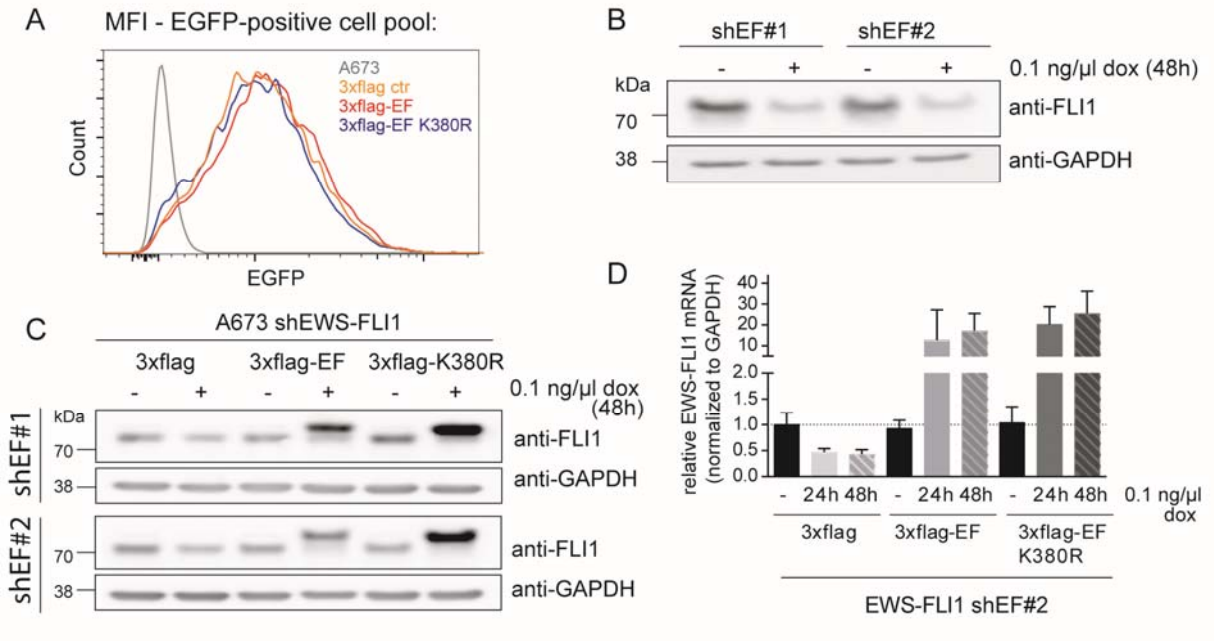
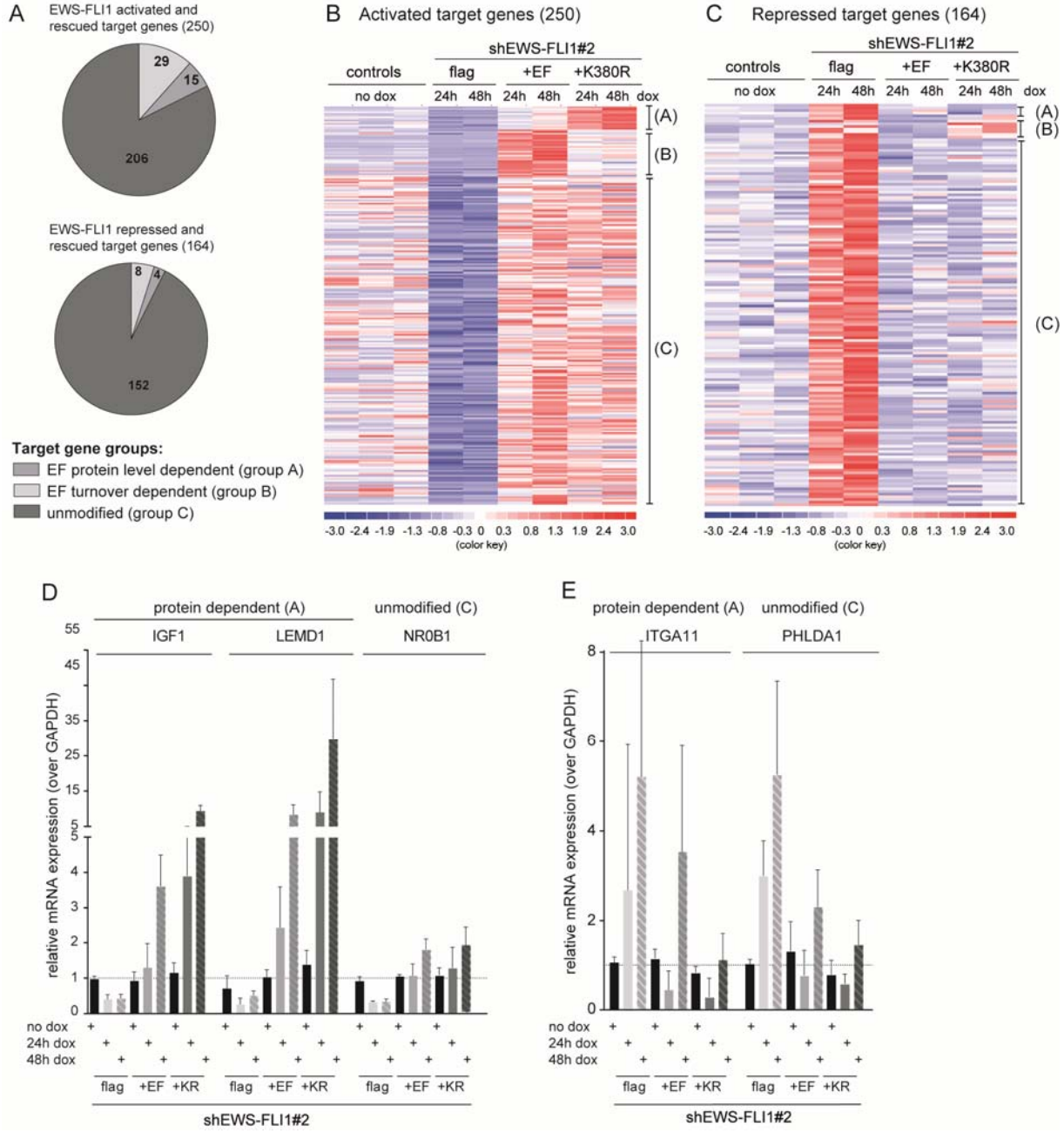


Figure 6



Proteasomal degradation of the EWS-FLI1 fusion protein is regulated by a single lysine residue

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Running title: EWS-FLI1 degradation is mediated by one lysine residue

Keywords: Ewing sarcoma, EWS-FLI1, ETS transcription factors, protein turnover, ubiquitin, target genes

Supplementary information

S1- supplementary information mass spectrometry

- Mass spectrometry peptide spectra for EWS-FLI1 K298, FLI1 K172 and K252
- Table with predicted and observed peptide mass
- Extended description of mass spectrometry sample preparation and analysis

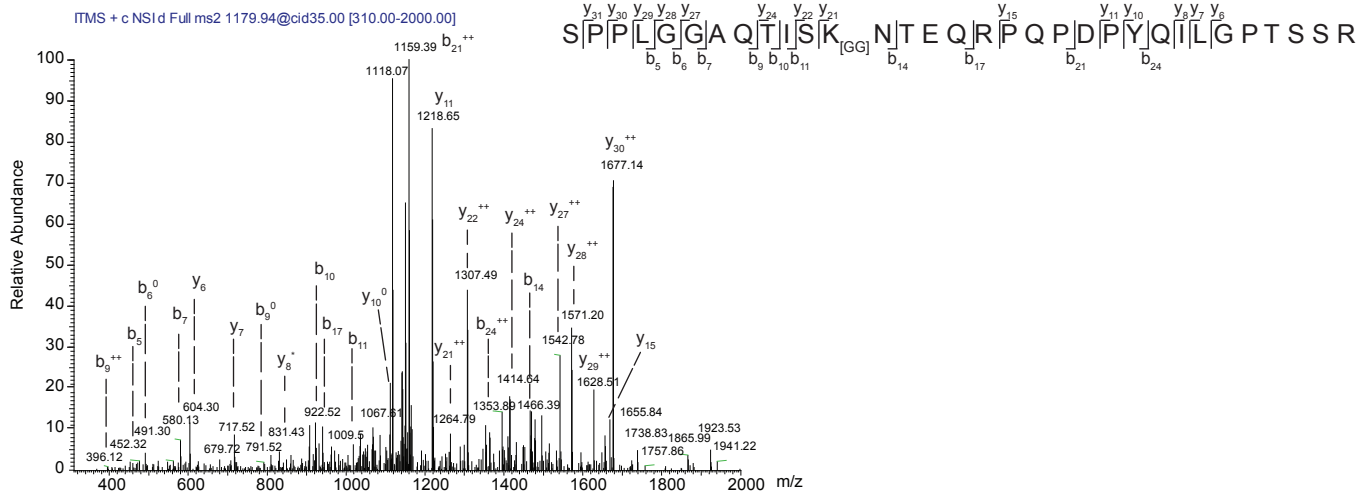
S2 - supplementary information microarray expression

- Extended description of microarray expression data analysis
- Lists of protein and turnover dependent target genes

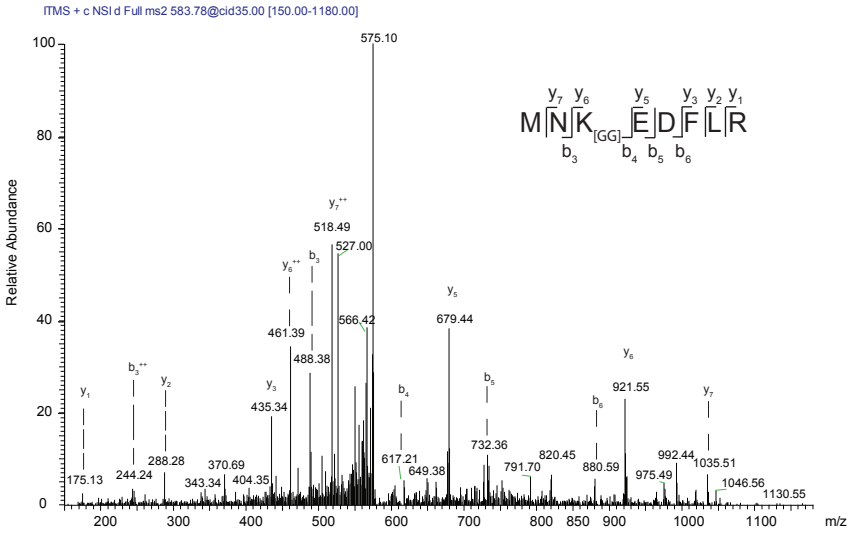
ST1 - supplementary information of plasmids, cloning primers and list of assays on demand

Supplementary S1 - Mass spectrometry

A EWS-FLI1 K298 peptide spectra



B FLI1 K172 peptide spectra



C FLI1 K252 peptide spectra

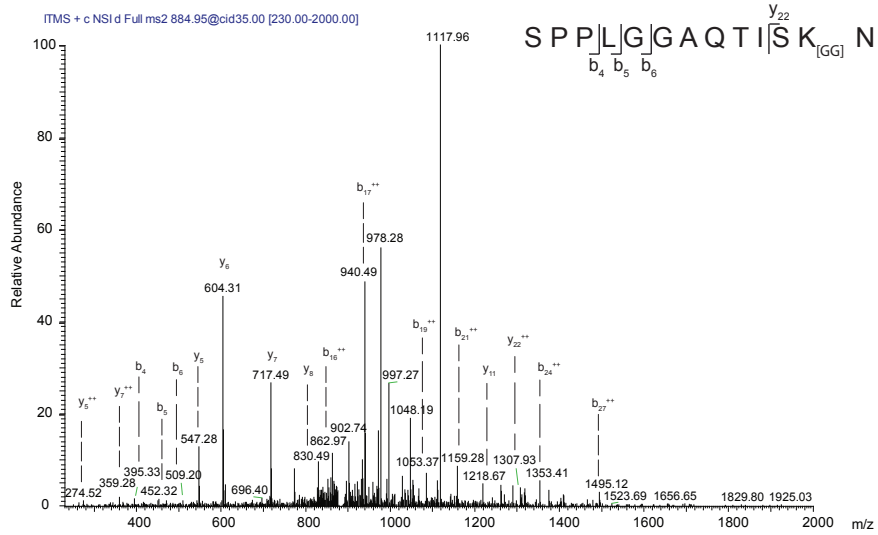


Figure:	Sample	Lysine residue	Residue number	Peptide sequence	Predicted mass	Observed mass
S1A:	EWS-FLI1	K298	287-318	SPPLGGAQTIS**K**NTEQRPQPDPYQILGPTSSR	3533.79	3533.78
F3A:	EWS-FLI1 R386N	K380	372-383	SKPNMNYD**K**LSR	1565.76	1565.75
S1B:	FLI1 wild type	K172	170-177	MN**K**EDFLR	1165.56	1165.55
S1C:	FLI1 wild type	K252	241-272	SPPLGGAQTIS**K**NTEQRPQPDPYQILGPTSSR	3533.79	3533.76

Mass spectrometry (MS) – extended description for sample preparation and analysis

Purification and sample preparation for MS.

Proteins were purified from HEK293T cells under denaturing conditions and separated by gel electrophoresis as described. The gel was stained with coomassie (Instant blue, Expedeon, Harston, UK) for 20min followed by washing with water for each 30min twice. Bands corresponding to the molecular weight of ubiquitinated EWS-FLI1 were excised from the gel and dried by speed vacuum for 10min. Digestion was performed with 50ng Trypsin (Promega, V511C) overnight at 37°C and stopped by addition of 0.2µl formic acid (FA). Peptides were extracted from the gel by adding twice 50% acetonitrile (ACN)- 5% trifluoroacetic acid (TFA).

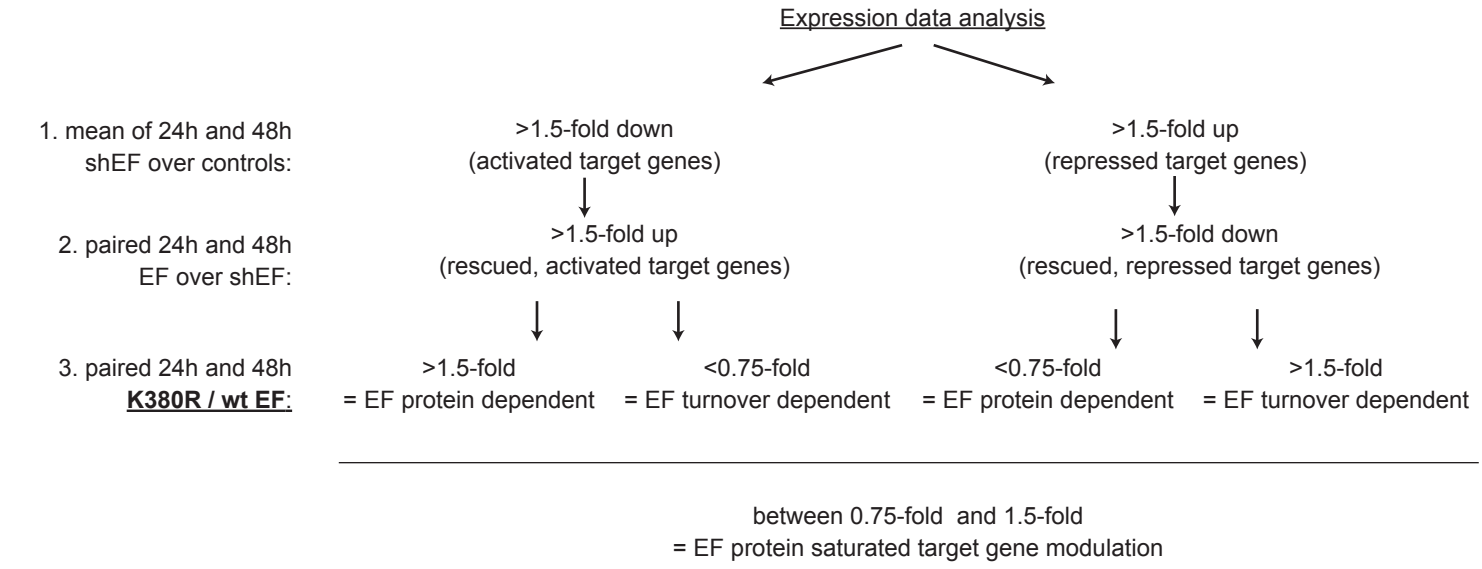
After drying by speed vacuum for 1h, peptides were cleaned with ZIP-TIP C18 tips (ThermoFisher Scientific AG) by resuspending in 3% ACN- 0.1% TFA and eluted with 60% ACN- 0.1% TFA twice. Final resuspension was in 3% ACN- 0.2% FA with 10mM citric acid before injection into an LTQ-OrbitrapVelos mass spectrometer (ThermoFisher Scientific AG) coupled to an EksigentNanoLC-Ultra 1D plus (Eksigent Technologies, Dublin, CA, USA). Solvent composition at the two channels was 0.1% FA for channel A and 0.1% FA, 99.9% ACN for channel B. Peptides were loaded on a self-made frit column (75µm × 150mm) packed with reverse phase C18 material (ReproSil-Pur 120 C18-AQ, 1.9µm, Dr. Maisch GmbH, Ammerbuch, Germany) and eluted at a flow rate of 250nl per min by a gradient from 3 to 30% of B in 56min. Full-scan MS spectra (300−1700 m/z) were acquired in profile mode at a resolution of 30'000 at 400 m/z, an accumulation gain control (AGC) of $1x10^6$ and a maximum injection time of 200ms. Collision induced dissociation (CID) MS/MS spectra were recorded in data dependent manner in the ion trap from the twenty most intense signals above a threshold of 1000, using a normalized collision energy of 35% and an activation time of 10ms. The AGC value for MS/MS analysis was set to $1x10^4$ (ion trap detection, 100ms injection time) and the isolation width was set to 2amu. Charge state screening was enabled and singly charge states were rejected. Precursor masses already selected for MS/MS were excluded for further selection for 45s and the exclusion window was set to 10ppm. The size of the exclusion list was set to a maximum of 500 entries. The samples were acquired using internal lock mass calibration on m/z 429.088735 and 445.120025.

Data analysis.

The raw-files from the mass spectrometer were converted into Mascot generic files (mgf) with Mascot Distiller software 2.4.3.3 (Matrix Science Ltd.,London, UK). The peak lists were searched using Mascot Server 2.3.02 against the forward UniProtKB/Swiss-Prot database for human, concatenated to a reversed decoyed FASTA database consisting of a total of 135,183 proteins with accessions in Gene Ontology compatible format and 260 common protein contaminants (NCBI taxonomy ID 9606, release date 2012-04-12). The protein sequence of EWS-FLI1 (B1PRL2_HUMAN) was included in the database.

The parameters for precursor tolerance and fragment ion tolerance were set to ± 5ppm and ± 0.8Da, respectively. Gly-Gly and oxidation (M) were set as variable modifications. Enzyme specificity was set to trypsin, allowing up to 1 missed cleavage. Peptides having an expectation value higher of 0.05 and/or a Mascot score lower of 20 were excluded. Only peptides with internal lysine modified by glycine-glycine were considered. For the localization of glycine-glycine modification, the results of Mascot Site Analysis were used. All the spectra of ubiquitinated peptides were manually validated.

Supplementary S2 - microarray expression analysis



S2 supplementary table: List of activated target ge nes which expression is either EWS-FLI1 protein sen sitive or turnover sensitive

EWS-FLI1 protein level sensitive

	Identifier	Gene Symbol	mRNA Accession	mRna - Description	Fold change (K380R / wild type EF)		Citation
1	17121292	SORD	ENST00000267814	ensembl_havana_transcript:known chromosome:GRCh38:15:45023104:45077185:1 ge	1,99	protein-coding	
2	16748814	LOC102724196	XR_432795	PREDICTED: Homo sapiens uncharacterized LOC102724196 (LOC102724196), ncRNA	2,23	non-coding RNA	
3	16802461	LINC00277	NR_026949	Homo sapiens long intergenic non-protein coding RNA 277 (LINC00277), long non-codi	2,06	non-coding RNA	Howarth et al. 2012
	17121206	LINC00277	NR_026949	Homo sapiens long intergenic non-protein coding RNA 277 (LINC00277), long non-codi	1,79	non-coding RNA	
4	16784564	---	NONHSAT037063	Non-coding transcript identified by NONCODE: Linc	1,86	non-coding RNA	
5	16769250	IGF1	NM_000618	Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1), transcript variant 4,	2,81	protein-coding	Cironi et al. 2008
6	16712944	KIAA1462	NM_020848	Homo sapiens KIAA1462 (KIAA1462), mRNA.	1,66	protein-coding	
7	16967875	PARM1	NM_015393	Homo sapiens prostate androgen-regulated mucin-like protein 1 (PARM1), mRNA.	1,56	protein-coding	
8	16754269	TRHDE	NM_013381	Homo sapiens thyrotropin-releasing hormone degrading enzyme (TRHDE), mRNA.	1,52	protein-coding	
9	16833184	LOC102724731	XR_424812	PREDICTED: Homo sapiens uncharacterized LOC102724731 (LOC102724731), transcr	1,94	non-coding RNA	
10	17126082	---	---	non-annotated	1,57	unknown	
11	17126124	---	---	non-annotated	1,57	unkonwn	
12	16690739	KCNA2	NM_001204269	Homo sapiens potassium voltage-gated channel, shaker-related subfamily, member 2 (I	1,72	protein-coding	
13	16939479	ENTPD3	NM_001248	Homo sapiens ectonucleoside triphosphate diphosphohydrolase 3 (ENTPD3), transcript	1,83	protein-coding	
14	16698489	LEMD1	NM_001001552	Homo sapiens LEM domain containing 1 (LEMD1), transcript variant 3, mRNA.	3,96	protein-coding	
15	16837348	MAP2K6	NM_002758	Homo sapiens mitogen-activated protein kinase kinase 6 (MAP2K6), mRNA.	2,04	protein-coding	

EWS-FLI1 turnover sensitive

	Identifier	Gene Symbol	mRNA Accession	mRna - Description	Fold change (K380R / wild type EF)	
1	16972108	APELA	ENST00000507152	havana:novel chromosome:GRCh38:4:164877004:164897523:1 gene:ENSG000002483	0,56	protein-coding
2	16949537	TP63	NM_001114978	Homo sapiens tumor protein p63 (TP63), transcript variant 2, mRNA.	0,57	protein-coding
3	16894044	LINC01249	NR_034134	Homo sapiens long intergenic non-protein coding RNA 1249 (LINC01249), long non-cod	0,56	non-coding RNA
4	16679349	KMO	NM_003679	Homo sapiens kynurenine 3-monooxygenase (kynurenine 3-hydroxylase) (KMO), mRNA	0,58	protein-coding
5	16777544	LINC00463	NR_120428	Homo sapiens long intergenic non-protein coding RNA 463 (LINC00463), long non-codi	0,57	non-coding RNA
6	17010005	BAI3	NM_001704	Homo sapiens brain-specific angiogenesis inhibitor 3 (BAI3), mRNA.	0,45	protein-coding
7	16909277	SPHKAP	NM_001142644	Homo sapiens SPHK1 interactor, AKAP domain containing (SPHKAP), transcript varian	0,38	protein-coding
8	16947022	RP11-454C18.1	OTTHUMT00000357	cdna:all chromosome:VEGA56:3:151637174:151657966:1 Gene:OTTHUMG000001598	0,51	unknown
9	16670185	LOC284561	AK023809	Homo sapiens cDNA FLJ13747 fis, clone PLACE3000276.	0,37	non-coding RNA
10	16674965	---	NONHSAT008377	Non-coding transcript identified by NONCODE: Linc	0,53	non-coding RNA
11	16896049	ALK	NM_004304	Homo sapiens anaplastic lymphoma receptor tyrosine kinase (ALK), mRNA.	0,62	protein-coding
12	16816962	SCNN1G	NM_001039	Homo sapiens sodium channel, non-voltage-gated 1, gamma subunit (SCNN1G), mRN	0,64	protein-coding
13	16851801	RNF125	NM_017831	Homo sapiens ring finger protein 125, E3 ubiquitin protein ligase (RNF125), mRNA.	0,50	protein-coding
14	17120752	C12orf55	ENST00000524981	havana:putative chromosome:GRCh38:12:96489587:96875555:1 gene:ENSG00000188	0,42	protein-coding
15	16780133	SLITRK6	NM_032229	Homo sapiens SLIT and NTRK-like family, member 6 (SLITRK6), mRNA.	0,36	protein-coding
16	16982264	MTNR1A	NM_005958	Homo sapiens melatonin receptor 1A (MTNR1A), mRNA.	0,43	protein-coding
17	17004536	LOC101928047	XR_249939	PREDICTED: Homo sapiens uncharacterized LOC101928047 (LOC101928047), ncRNA	0,52	non-coding RNA
18	17016390	HIST1H2BG	NM_003518	Homo sapiens histone cluster 1, H2bg (HIST1H2BG), mRNA.	0,65	protein-coding
19	17125422	CD99P1	NR_033381	Homo sapiens CD99 molecule pseudogene 1 (CD99P1), transcript variant 2, non-coding	0,48	non-coding RNA
20	16975642	GABRA4	NM_000809	Homo sapiens gamma-aminobutyric acid (GABA) A receptor, alpha 4 (GABRA4), transc	0,34	protein-coding
21	17063507	KDM7A	NM_030647	Homo sapiens lysine (K)-specific demethylase 7A (KDM7A), mRNA.	0,53	protein-coding
22	16733038	RNU6-321P	ENST00000410912	ncrna:known chromosome:GRCh38:11:125277552:125277657:1 gene:ENSG00000222	0,20	non-coding RNA
23	16666055	CTH	ENST00000346806	ensembl_havana_transcript:known chromosome:GRCh38:1:70411383:70439204:1 gen	0,61	protein-coding
24	16685593	---	NONHSAT002513	Non-coding transcript identified by NONCODE: Linc	0,27	non-coding RNA
25	17005072	RNU6-522P	ENST00000364497	ncrna:known chromosome:GRCh38:6:15314920:15315026:1 gene:ENSG00000201367	0,39	non-coding RNA
26	16669278	FAM46C	NM_017709	Homo sapiens family with sequence similarity 46, member C (FAM46C), mRNA.	0,62	protein-coding
27	17115913	---	NONHSAT139285	Non-coding transcript identified by NONCODE: Linc	0,64	non-coding RNA
28	16669105	MAB21L3	NM_152367	Homo sapiens mab-21-like 3 (C. elegans) (MAB21L3), mRNA.	0,45	protein-coding
29	17013657	ULBP1	NM_025218	Homo sapiens UL16 binding protein 1 (ULBP1), mRNA.	0,31	protein-coding

S2 supplementary table: List of repressed target ge nes which repression is either EWS-FLI1 protein sen sitive or turnover sensitive

EWS-FLI1 protein level sensitive

	Identifier	Gene Symbol	mRNA Accession	mRna - Description	Fold change (K380R / wild type EF)	
1	17077502	TOX	NM_014729	Homo sapiens thymocyte selection-associated high mobility group box (TOX), mRNA.	0,60	protein-coding
2	16888270	ITGA4	NM_000885	Homo sapiens integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor) (IT	0,58	protein-coding
3	16778241	POSTN	NM_001135934	Homo sapiens periostin, osteoblast specific factor (POSTN), transcript variant 2, mRNA	0,52	protein-coding
4	16811085	ITGA11	NM_001004439	Homo sapiens integrin, alpha 11 (ITGA11), mRNA.	0,63	protein-coding

EWS-FLI1 turnover sensitive

	Identifier	Gene Symbol	mRNA Accession	mRna - Description	Fold change (K380R / wild type EF)	
1	16878731	EHD3	NM_014600	Homo sapiens EH-domain containing 3 (EHD3), mRNA.	1,69	protein-coding
2	16821377	CDH13	NM_001220488	Homo sapiens cadherin 13 (CDH13), transcript variant 2, mRNA.	1,55	protein-coding
3	16671457	IL6R	NM_000565	Homo sapiens interleukin 6 receptor (IL6R), transcript variant 1, mRNA.	1,56	protein-coding
4	17117110	CD24	BC064619	Homo sapiens CD24 molecule, mRNA (cDNA clone MGC:75043 IMAGE:5591617), cor	10,97	protein-coding
5	17020258	BMP5	NM_021073	Homo sapiens bone morphogenetic protein 5 (BMP5), mRNA.	2,01	protein-coding
6	16805287	MIR3175	NR_036136	Homo sapiens microRNA 3175 (MIR3175), microRNA.	1,96	non-coding RNA
	17118970	MIR3175	NR_036136	Homo sapiens microRNA 3175 (MIR3175), microRNA.	1,96	non-coding RNA
7	16979613	---	NONHSAT098206	Non-coding transcript identified by NONCODE: Linc	1,67	non-coding RNA
8	17044046	RPL23P8	NR_026673	Homo sapiens ribosomal protein L23 pseudogene 8 (RPL23P8), non-coding RNA.	1,65	non-coding RNA

Supplementary table ST1: List of plasmids and primers used for cloning and mutagensis + list of assay on demand for qRT-PCR

General plasmids:

pCMV-3xflag	Sigma Aldrich	
EWS-FLI1	H. Kovar, Children's Cancer Research Institute, Vienna, Austria	
EWSR1	H. Gehring, University of Zurich, Switzerland	
FLI1	J. Ghysdael, Institute Curie, France (Bailly et al. 1994 MolCellBiol)	
Primer name	Primer sequence 5'-3'	Cloning strategy
pCMV-3xflag-EWS FW	GACAAGCTTGCGGCCGCGCGTCCACGGATTACAGTACC	EWS-FLI1, EWS into pCMV-3xflag
pCMV-3xflagFLI1 FW	GACAAGCTTGCGGCCGCAAACCCTTCTTATGACTCAGTCAGAAG	FLI1 into pCMV-3xflag
pCMV-3xflag-FLI1 RS	GATGAATTCGCGGCCGCTCACTAGTAGTAGCTGCCTAAGTGTGA	EWS-FLI1, FLI1 into pCMV-3xflag
pCMV-3xflag-EWS RS	GATGAATTCGCGGCCGCTCACTAGTAGGGCCGATCTCTG	EWS into pCMV-3xflag

Global Protein Stability - plasmids:

pR-EF1-cDNA	Cellecta Inc.	
MSCV-CMV-DsRed-IRES-EGFP-DEST	Addgene #41941	
MSCV-CMV-DsRed-IRES-d1EGFP	Addgene #41942	
MSCV-CMV-DsRed-IRES-d4EGFP	Addgene #41943	
MSCV-CMV-DsRed-IRES-d24EGFP	Addgene #41944	
Primer name	Primer sequence 5'-3'	Cloning strategy
RIG FW	TAG AGC TAG CGA ATT CAT GGC CTC CTC CGA GGA C	#41941/2/3/4 into pR-EF1
RIG RS	ATT TAA ATT CGA ATT CTT GGC CAG ATC TGA GTC C	#41941 into pR-EF1
d1/4 EGFP RS	ATT TAA ATT CGA ATT CCT ACA CAT TGA TCC TAG CAG	#41942/3 into pR-EF1
d24 EGFP RS	ATT TAA ATT CGA ATT CTT ACT TGT ACA GCT CGT CCA TG	#41944 into pR-EF1
RIG EF FW	TCT GGC CAG AAA TTC GAA ATG CGT CCA CGG ATT ACA GTA CC	EWS/FLI1, EWS in pR-EF1-RIG
RIG EF RS	TCC GAT TTA AAT TCG AAT TAC TAG TAG TAG CTG CCT AAG TGT G	EWS/FLI1, FLI1 in pR-EF1-RIG
RIG EWS RS	TCC GAT TTA AAT TCG AAT TAC TAG TAG GGC CGA TCT CTG C	EWS in pR-EF1-RIG
RIG FLI RS	TCT GGC CAG AAA TTC GAA ATG ACG GGA CTA TTA AGG AGG C	FLI1 in pR-EF1-RIG

Exchange cell lines - plasmids:

Cellecta shEF#1	Target sequence 5' ATAGAGGTGGGAAGCTTATAA 3'	
Cellecta shEF#2	Target sequence 5' CGTCATGTTCTGGTTTGAGAT 3'	
pINDUCER21 (ORF-EG)	Addgene #46948	
Primer name	Primer sequence 5'-3'	Cloning strategy
pInd 3xf EF FW	GCG GCC CCG AAC TAG TAT GGA CTA CAA AGA CCA TGA C	3xflag-EWS-FLI1, 3xflag into pInducer21
pInd 3xf EF RS	TCG TAT GGG TAT TCG AAC TAC TAG TAG TAG CTG CCT AAG	3xflag-EWS-FLI1 into pInducer21
pInd 3xflag RS	GTA TGG GTA TTC GAA CTA CTA TGC GGC CGC AAG CTT GTC	3xflag into pInducer21

Primer for site directed mutagenesis

Primer name	Primer sequence 5'-3'	Mutagenesis
EF K298R FW	GGG GCA CAA ACG ATC AGT AGG AAT ACA GAG CAA CGG CCC	EWS-FLI1 and FLI1 in K298/252>R
EF K298R RS	GGG CCG TTG CTC TGT ATT CCT ACT GAT CGT TTG TGC CCC	EWS-FLI1 and FLI1 in K298/252>R
EF K380R FW	AAG CCC AAC ATG AAT TAC GAC AGG CTG AGC CGG GCC CTC	EWS-FLI1 and FLI1 in K380/334>R
EF K380R RS	GAG GGC CCG GCT CAG CCT GTC GTA ATT CAT GTT GGG CTT	EWS-FLI1 and FLI1 in K380/334>R
FLI1 K172R FW	GGA TGG CAA GGA ACT GTG TAA AAT GAA CAG GGA GGA C	FLI1 in K172>R
FLI1 K172 RS	GTC CTC CCT GTT CAT TTT ACA CAG TTC CTT GCC ATC C	FLI1 in K172>R
EF R386N FW	CTGAGCCGGGCCCTCAATTATTACTATG	EWS-FLI1 in R386>N
EF R386N RS	CATAGTAATAATTGAGGGCCCGGCTCAG	EWS-FLI1 in R386>N

List of commercially available assay on demand for qRT-PCR

Gene:	Applied Biosystems assays on demand:
EWS-FLI1	Hs03024807_ft
GAPDH	Hs04420697_g1
IGF1	Hs01547656_m1
LEMD1	Hs01077215_m1
NR0B1	Hs00230864_m1
ITGA11	Hs00201927_m1
PHLDA1	Hs00378285_g1

Proteasomal degradation of the EWS-FLI1 fusion protein is regulated by a single lysine residue

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